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PATENT
Customer No. 22,852
Attorney Docket No. **02481.1751**

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:	)	
	)	
Cordula HOPMANN, et al.	)	Group Art Unit: 1623
	)	
Application No.: 09/966,109	)	Examiner: Elli Peselev
	)	
Filed: October 1, 2001	)	
	)	
For: CITRULLIMYCINES, A PROCESS FOR THEIR PRODUCTION AND THEIR USE AS PHARMACEUTICALS	)	

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir

DECLARATION UNDER 37 C.F.R. § 1.132

I, Dr. Joachim Manfred Wink, declare and state that:

1. I am a citizen of Germany, residing at Magdeburger Strasse 14, 63322 Rödermark, Germany.

2. In 1985, I obtained a degree of Doctor of Science (Dr. phil. nat.) at the Faculty of Biology of Johann Wolfgang Goethe University in Frankfurt.

3. In 1985, I was a Laboratory Manager at Hoechst AG, Department of Biotechnology (central research). In 1992, I moved to Pharmaceutical Research at Hoechst AG with the task of assembling, maintaining and administering a collection of strains comprising about 20,000 isolates for natural substance screening. In 1997,

Hoechst AG became Hoechst Marion Roussel Deutschland GmbH. In 2000, a merger with Rhône-Poulenc created Aventis Pharma; Aventis Pharma Deutschland GmbH belongs to Aventis Pharma. After this merger, there are currently more than 60,000 isolates of Actinobacteria in the collection.

4. The Experiments described in this declaration were performed by me or under my direct supervision, unless otherwise indicated.

5. The strain designations and deposit numbers of the strains used in the comparisons reported in this declaration are listed in Table 1.

Table 1: Strains of the genus Streptomyces used for direct comparative studies

Species	Strain Designation	Deposit No.	Document
Streptomyces spp.	ST 104890	DSM 4200	US 5,252,472
Streptomyces spp.	FH 6387	DSM 4211	US 5,252,472
Streptomyces spp.	FH 6388	DSM 4349	US 5,252,472
Streptomyces spp.	FH 6389	DSM 4355	US 5,252,472
Streptomyces spp.	ST 101396 (identical to HAG 012114)	DSM 13309	USSN 09/966,109

6. Morphology and Colony Growth

For comparison of the morphology and colony growth, the strains were cultivated on agar media: ISP 2 is Medium 2 in Shirling and Gottlieb, ISP 3 is Medium 3 in Shirling and Gottlieb, and so forth. E. B. Shirling and D. Gottlieb, *Methods for Characterization of Streptomyces Species*, 16 International Journal of Systematic Biology, 313, 315-318 (1966). This reference is also referred to as the International Streptomyces Project. Growth, color of the substrate mycelium, color of the soluble exopigment, and color of aerial mycelium have been determined; see Table 2 and strain cards in Appendix B. The assessment of growth was performed according to Shirling and Gottlieb. The color

determination was made according to the RAL color code (RAL is Deutsches Institut für Gütesicherung und Kennzeichnung e.V. - Reichsausschuss für Lieferbedingungen). Color photographs showing colony color and growth morphology on media ISP 2 and ISP 3 are provided in Figures 1 and 2 in Appendix C. DSM 13309 has a characteristic mouse grey aerial mycelium and is the only strain which is melanin positive on the tyrosine containing medium. The soluble exopigment has color in Suter medium (Suter, M.A., 1978, Isolierung von Melanin negativen Mutanten aus Streptomyces glaucescens, Dissertation, ETH Zürich 6276), and the pigment formation is not correlated with melanin.

Table 2. Colony characteristics of *Streptomyces spp* DSM 4200, *Streptomyces spp* DSM 4211, Streptomyces *spp* DSM 4349, *Streptomyces spp.* DSM 4355 and *Streptomyces spp.* DSM 13309

Culture medium	**Strains**				
	DSM 4200	DSM 4211	DSM 4349	DSM 4355	DSM 13309
ISP 2	SM saffron yellow AM beige red SP none	SM fawn brown AM beige red SP none	SM maize yellow AM squirrel grey SP none	SM sand yellow AM grey white brown SP ochre brown	SM sand yellow AM mouse grey SP none
ISP 3	SM red orange AM beige red SP none	SM brown beige AM beige red SP none	SM sand yellow AM squirrel grey SP none	SM signal yellow AM grey white SP light pink	SM ivory AM mouse grey SP none
ISP 4	SM oxide red AM beige SP none	SM beige AM grey white SP clay brown	SM copper brown AM squirrel grey SP none	SM nut brown AM grey white SP none	SM ivory AM mouse grey SP none
ISP 5	SM sand yellow AM none SP none	SM ivory AM none SP none	SM sand yellow AM none SP none	SM beige AM none SP none	SM light ivory AM none SP none
ISP 6	SM honey yellow AM none SP none	SM sand yellow AM none SP none	SM sand yellow AM none SP beige grey	SM sand yellow AM none SP none	SM sand yellow AM none SP none
ISP 7	SM brown beige AM none SP none	SM sand yellow AM none SP none	SM sand yellow AM none SP none	SM sand yellow AM none SP none	SM beige AM none SP mahogany brown

Formation and color of: SM, substrate mycelium; AM, aerial mycelium; SP, soluble exopigment

7. Carbohydrate Utilization

The method described in the International Streptomyces Project was used to determine carbohydrate utilization. *Id.* at 335-336. All strains show a different pattern. Only DSM 4349 and DSM 4355 show similarity in most of the carbohydrates; the only difference is the use of D-xylose by strain DSM 4349.

Table 3. Utilization of carbohydrates by *Streptomyces spp* DSM 4200, *Streptomyces spp* DSM 4211, *Streptomyces spp* DSM 4349, *Streptomyces spp.* DSM 4355 and *Streptomyces spp.* DSM 13309

Carbohydrate	**Strains**				
	DSM 4200	DSM 4211	DSM 4349	DSM 4355	DSM 13309
glucose	+	+	+	+	+
L-arabinose	+	-	+	+	+
sucrose	(+)	-	+	+	-
D-xylose	(+)	-	+	(+)	-
I-inositol	(+)	-	+	+	-
mannitol	(+)	-	+	+	+
D-fructose	+	-	+	+	+
rhamnose	(+)	-	+	+	+
raffinose	(+)	-	+	+	+

Growth not better than the negative control (basal medium without a carbon source) -

Growth equal to or better than the positive control (basal medium with glucose) +

Growth better than the negative control, but less than the positive control (+)

8. Enzymatic Activities and Physiological Characteristics

The enzymatic activities and physiological characteristics of the strains were identified with API ZYM and API 20E test kits purchased from bioMerieux and used according to the manufacturer's instructions. Table 4 shows that characteristic differences exist between all strains.

Table 4. Enzymatic activities and physiological characteristics of Streptomyces spp DSM 4200, Streptomyces spp DSM 4211, Streptomyces spp DSM 4349, Streptomyces spp. DSM 4355 and Streptomyces spp. DSM 13309

	Strip Test	Strains				
		DSM 4200	DSM 4211	DSM 4349	DSM 4355	DSM 13309
API ZYM	Phosphatase alkaline	(+)	+	-	+	+
	Esterase (C4)	-	+	+	(+)	+
	Esterase Lipase (C8)	+	+	+	-	+
	Lipase (C14)	-	+	-	-	-
	Leucine arylamidase	+	+	+	+	+
	Valine arylamidase	+	+	+	+	+
	Cystine arylamidase	+	+	+	+	+
	Trypsin	+	+	+	+	+
	Chymotrypsin	+	+	+	+	+
	Phosphatase acid	+	+	-	+	+
	Naphthol-AS-BI-phosphohydrolase	+	+	+	+	+
	α galactosidase	-	-	(+)	+	+
	ß galactosidase	+	-	-	+	+
	ß glucuronidase	-	-	(+)	-	-
	α glucosidase	+	+	+	+	+
	ß glucosidase	-	-	-	+	+
	N-acetyl-ß-glucoseamidase	+	+	-	+	+
	α mannosidase	+	+	-	+	+
	α fucosidase	-	-	-	-	-
API 20E	beta-galactosidase	(+)	-	+	+	+
	arginine dihydrolase	+	+	+	+	+
	lysine decarboxylase	-	+	-	+	-
	ornithine dacarboxylase	+	(+)	+	(+)	+
	citrate utilization	+	+	+	+	+
	H_2S production	-	-	-	-	-
	Urease	+	+	+	+	+
	tryptophane desaminase	-	-	-	-	-
	indole production	-	-	-	-	-
	acetoin production	-	-	+	+	-
	gelatinase	+	+	+	+	+

9. Fatty Acid Analyses

The fatty acid analyses (1) show that DSM 13309 is different from the other strains and (2) confirms that DSM 13309 is of the Streptomyces genus. The analyses of the cell wall fatty acids were carried out by the HP/MIDI system using the modified extraction procedure of Aretz *et al.* HP5898A Bakterien-Identifizierungssystem, Hewlett Packard User Manual; L. Miller, *Gas-Liquid Chromatography of Cellular Fatty Acids as a Bacterial Identification Acid*, Hewlett Packard, Application Note 228-37; J. P. Siegel A. R. Smith and R. J. Novak, *Comparison of the Cellular Fatty Acid Composition of a Bacterium Isolated from a Human and Alleged to be Bacillus sphaericus with That of Bacillus sphaericus Isolated from a Mosquito Larvicide*, 63(3) Applied and Environmental Microbiology, 1006-1010 (1997) (see page 1007, left col.); W. Aretz, J Meiwes, G. Seibert, G. Vobis and J. Wink, *Friulimicins: Novel Lipopeptide Antibiotics with Peptidoglycan Synthesis Inhibiting Activity from Actinoplanes friuliensis sp. nov. I. Taxonomic Studies of the Producing Microorganism and Fermentation,* 53(8) J. Antibiotics, 807-815 (2000).

These analyses show that all five strains have the characteristic iso and ant-eiso branched fatty acids of the genus Streptomyces; see Table 5. Detailed fatty acid profiles are provided in Appendix D.

Table 5. Fatty acid patterns of *Streptomyces spp* DSM 4200, *Streptomyces spp* DSM 4211, *Streptomyces spp* DSM 4349, *Streptomyces spp.* DSM 4355 and *Streptomyces spp.* DSM 13309

Fatty Acid	Strains				
	DSM 4200	DSM 4211	DSM 4349	DSM 4355	DSM 13309
14:0 Iso	9.04	8.82	6.37	7.96	8.84
15:0 Iso	4.72	6.85	9.47	14.00	7.41
15:0 Anteiso	16.06	21.28	14.25	21.02	22.26
16:0 Iso	28.98	20.75	15.84	28.04	23.04
16:0	7.94	8.48	13.92	20.30	8.74
17:0 Anteiso	3.95	6.58	3.92	8.67	6.18

10. Ribotyping

Ribotyping is a molecular method based on the analysis of restriction fragment length polymorphs of ribosomal RNA genes. The RiboPrinter system combines the molecular processing steps for ribotyping in a stand-alone, automated instrument. These steps include: cell lysis, digestion of chromosomal DNA with restriction enzymes (kits for *Eco*RI and *Pvu*II are available, but other restriction enzymes can be used), separation of fragments by electrophoresis, transfer of DNA fragments to a nylon membrane, hybridization of the DNA fragments to a probe generated from the *rrn*B operon from *E. coli,* chemoluminescence detection of the probe attached to the fragments containing *rrn* operon sequences, image detection and computerized analysis of RiboPrint patterns. With the aid of RiboPrint pattern, microorganism strains can be differentiated from one another. Some references in Appendix A relate to Ribotyping.

Standardized, automated Ribotyping was not performed by me or under my supervision but was performed by the German Culture Collection (DSMZ) using the Qualicon™ RiboPrinter system. The restriction enzyme *Pvu*II was used to digest the

chromosomal DNA of the strains ST 101396 = DSM 13309 (ID 03-1309), ST 104890 = DSM 4200 (ID 03-1310), DSM 4349 (ID 03-1311), DSM 4355 (ID 03-1312) and DSM 4211 (ID 03-1313). The RiboPrints of the ribotyping analysis can be found in Appendix E. A summary of the results obtained from the RiboPrinter System follows:

a. The patterns of the strains ST 101396 = HAG 012114 = DSM 13309 (ID 03-1309), ST 104890 = DSM 4200 (ID 03-1310), DSM 4349 (ID 03-1311), DSM 4355 (ID 03-1312) and DSM 4211 (ID 03-1313) differ from each other.

b. Strain ST 101396 = DSM 13309 (ID 03-1309) was automatically correlated by the DuPont Identification Library as *Streptomyces rutgersensis* subsp. *castelarensis.* The respective entry of the Identification Library is based on strain *Streptomyces rutgersensis* subsp. *castelarensis* DSM 40830^T. The comparative RiboPrint of DSM13309 and DSM 40830^T shows 92% homology. Since greater than 99% homology is needed to show identical subspecies, DSM13309 and DSM 40830^T are different subspecies. Further, DSM 40830^T is not known to produce Citrullimycines.

c. Strain DSM 4349 (ID 03-1311) was automatically correlated by the DuPont Identification Library as *Streptomyces coeruleorubidus.* The respective entry of the Identification Library is based on strain *Streptomyces coeruleorubidus* DSM 40145^T.

d. The strains ST 104890 = DSM 4200 (ID 03-1310), DSM 4355 (ID 03-1312) and DSM 4211 (ID 03-1313) did not show similarities higher than 0.85 to entries of the DuPont Identification Library and were therefore not identified automatically. The pattern of strain ST 104890 = DSM 4200 (ID 03-1310) shows the highest similarity to this of *Streptomyces fradiae* DSM 41634 (0.69) within the DSMZ internal database.

The pattern of strain DSM 4355 (ID 03-1312) shows the highest similarity to those of *Streptomyces iakyrus* DSM 40482^T (0.65) and *Streptomyces crystallinus* DSM 40945^T (0.64) of the DSMZ database. The pattern of strain DSM 4211 (ID 03-1313) shows the highest similarity of those of *Streptomyces griseoloalbus* DSM 40468^T (0.77) and *Streptomyces netropsis* DSM 40093 (0.74) of the DSMZ database.

11. The above results clearly and unambiguously demonstrate that DSM 13309 is a different species than the others listed above, and that, in light of this comparison, DSM 13309 is a novel microorganism.

12. I declare further that all statements made herein (including the Appendices) of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Date: 22.04.2004

By: Joachim M. Wink

Dr. Joachim Manfred Wink

Appendices

A. Related References

B. Strain Cards Showing Additional Details of Morphological and Physiological Characteristics

C. Figures 1 and 2 Showing Colony Color and Growth

D. Details of the Fatty Acid Analyses

E. RiboPrints of the Ribotyping Analyses

Appendix A

Related References

HP5898A Bakterien-Identifizierungssystem, Hewlett Packard User Manual.

Riboprinting, printed from http://homepages.lshtm.ac.uk/entamoeba/riboprnt.htm, on March 9, 2004.

W. Aretz, J Meiwes, G. Seibert, G. Vobis and J. Wink, *Friulimicins: Novel Lipopeptide Antibiotics with Peptidoglycan Synthesis Inhibiting Activity from Actinoplanes friuliensis sp. nov. I. Taxonomic Studies of the Producing Microorganism and Fermentation,* 53(8) J. Antibiotics, 807-815 (2000).

J. L. Bruce, *Automated System Rapidly Identifies and Characterizes Microorganisms in Food,* 50(1) Food Technology, 77-81 (1996).

J. L. Bruce, R. J. Hubner, E. M. Cole, C. I. McDowell and J. A. Webster *Sets of EcoRI fragments containing ribosomal RNA sequences are conserved among different strains of Listeria moncytogenes,* 92 Proc. Natl. Acad. Sci. USA, 5229-5233 (1995).

L. Miller, *Gas-Liquid Chromatography of Cellular Fatty Acids as a Bacterial Identification Acid*, Hewlett Packard, Application Note 228-37.

E. B. Shirling and D. Gottlieb, *Methods for Characterization of Streptomyces Species*, 16 International Journal of Systematic Biology, 313, 315-318 (1966); This reference is also referred to as the International Streptomyces Project.

J. P. Siegel A. R. Smith and R. J. Novak, *Comparison of the Cellular Fatty Acid Composition of a Bacterium Isolated from a Human and Alleged to be Bacillus sphaericus with That of Bacillus sphaericus Isolated from a Mosquito Larvicide*, 63(3) Applied and Environmental Microbiology, 1006-1010 (1997).

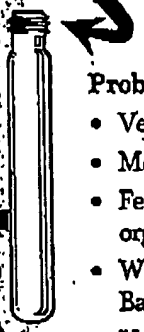
J. T. Tang, D. Cleland and D. Emerson, *Characterization of Bacteria by Ribotyping*, 23(1) ATCC Connection, 1 (2003).

J. A. Webster, T. L. Bannerman, R. J. Hubner, D. N. Ballard, E. M. Cole, J. L. Bruce, F. Fiedler, K. Schubert, and W. E. Kloos, *Identification of the Staphylococcus sciuri Species Group with EcoRi Fragments Containing rRNA Sequences and Description of Staphylococcus vitulus sp. nov.,* 44(3) Int. Journ. Sys. Bact., 454-460 (1994).

m Verfahren

ndard-Kulturverfahren

Jnbekannte Kultur auf
\gar-Platte streichen
Jnter gleichbleibenden
3edingungen inkubieren
Zellen in Gefäß mit Schraubverschluß
geben

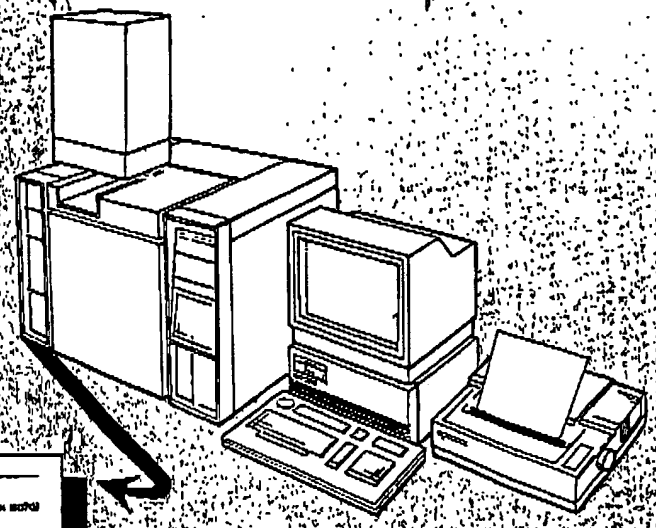


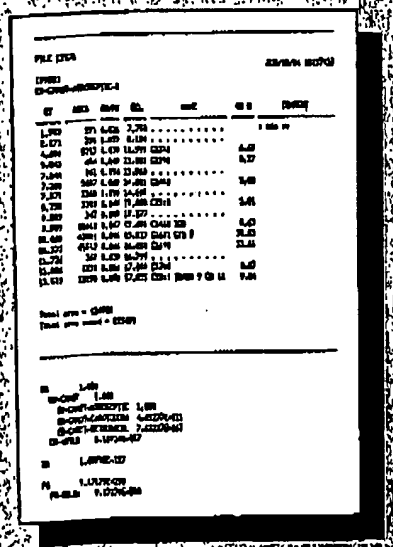
Probenvorbereitung in einem einzigen Reagenzglas

- Verseifung zur Freisetzung der Fettsäuren
- Methylierung zur Erhöhung der Flüchtigkeit
- Fettsäurenextraktion aus der wässrigen in eine organische Phase
- Waschen der organischen Phase mit einer anorganischen Base
- Umfüllen in Probenflaschen mit Septumverschluß

llautomatische Analyse

Protokollieren des Probennamens und
des Identifizierungscodes
Automatische Systemkalibrierung
Hochauflösende Gaschromatographie
Bibliotheksvergleich





Ergebnisse

- Fettsäurezusammensetzung
- Bestmögliche Übereinstimmung der Gattung, des Stammes und des Unterstammes mit den Bibliotheksdaten
- Güte der Übereinstimmung durch Indexziffer

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s Bakterien-Identifizierungssystem HP 5898A ist ein nputergesteuertes automatisches System zur genauen ntifizierung von Bakterien, Hefe- und Schimmelpilzen wie anderer Mikroorganismen. Das Herzstück des stems ist die hochauflösende gaschromatographische alyse der Zellwandlipide in Verbindung mit einer nputergestützten Bibliothek für bekannte Stämme.

eites Mikrobenspektrum

Schnelle und zutreffende Artbestimmung
Nach Isolieren des Mikroorganismus kann innerhalb von zwei Stunden die Identifizierung erfolgen – häufig bis hin zu Unterstämmen. In Verbindung mit einer systematischen Probenvorbereitung ist das System in der Lage, pro Tag bis zu fünfzig Mikroorganismen zu identifizieren.

Identifizierung ungewöhnlicher Mikroorganismen
Die Bibliothek enthält Daten vieler Organismen, die mit den üblichen biochemischen Verfahren nur schwer zu identifizieren sind.

Untersuchungen in Industrie, Landwirtschaft und klinischem Bereich
In der Bibliothek sind auch die Daten von Organismen enthalten, die bei industriellen Verfahren, in der Sterilitätsprüfung, für die Trinkwasserqualität, in der Landwirtschaft und der medizinischen Forschung sowie auf anderen Gebieten von Bedeutung sind.

ewährte Technik

Stabile Fettsäuren-„Fingerprints"
Langjährige Forschungsarbeiten haben ergeben, daß die qualitative und quantitative Zusammensetzung der Zellwand-Fettsäuren ein stabiles phänotypisches Erkennungsmerkmal des jeweiligen Bakterienstamms darstellt.

Korrelation mit der entsprechenden DNA-Struktur
Die Zellwand-Fettsäuren entsprechen der im Zellkern enthaltenen genetischen Information und werden durch den Informationsgehalt von Plasmiden nicht beeinflußt, so daß die Fettsäurenprofile mit den geltenden taxonomischen Festlegungen gut korrelieren.

Überlegenheit gegenüber der biochemischen Identifizierung
Der HP 5898A kann mehr als fünfzig Zellwand-Fettsäuren identifizieren. Das System bietet somit eine ausreichende Grundlage für eine Bibliothekssuche mit Mustererkennung.

Einfach und sicher

- **Ein Verfahren für alle Mikroorganismen**
  Für alle Mikroorganismen kann dasselbe grundlegende Vorbereitungsverfahren angewandt werden. Der Schulungsaufwand ist deshalb gering. Das Risiko einer falschen Identifizierung aufgrund unsachgemäßer Probenvorbereitung wird ausgeschlossen.
- **Probenvorbereitung in einem einzigen Reagenzglas**
  Die Probenvorbereitung von der Reinkultur bis hin zur chromatographiefähigen Probe erfolgt in einem einzigen Reagenzglas. Dadurch werden Probleme vermieden, die mit der Handhabung und der Weiterverfolgung der Probe verbunden sind.
- **Bakterienabtötung als erster Schritt**
  Die weitgehend vereinfachte Probenhandhabung stellt einen wesentlichen Beitrag zu höherer Sicherheit im Labor dar. Schon mit dem ersten Vorbereitungsschritt wird der Mikroorganismus abgetötet. Das ist vor allem beim Umgang mit möglicherweise pathogenen Keimen wichtig.
- **Computergestützte Analyse und Identifizierung**
  Sobald die Probenflasche in den automatischen Probengeber eingesetzt und vom Computer protokolliert worden ist, erfolgt der Analyse- und Identifizierungsprozeß vollautomatisch. Die Festlegung der Probenfolge, die Systemkalibrierung und das Ausdrucken der Ergebnisse werden ohne jedes weitere Zutun des Anwenders durchgeführt.

Die Vorteile von Hewlett-Packard

- **Kostengünstiger als die biochemische Identifizierung**
  Die Identifizierungskosten sind günstiger als bei der biochemischen Identifizierung mit Hilfe von Teststreifen. Sie betragen nur einen Bruchteil der Kosten der Identifizierung in einem Speziallabor.
- **Ständiger Ausbau der Bibliothek**
  Die Bakterienbibliothek wächst auch weiterhin in dem Maße an, wie neue Mikroorganismen isoliert und taxonomisch klassifiziert werden. HP liefert regelmäßig entsprechend den Bedürfnissen des Mikrobiologen aktuelle Nachträge für die Bibliothek.
- **Weltweiter HP-Service**
  In einem engmaschigen Servicenetz stehen geschulte Verkaufs- und Serviceingenieure zur Verfügung und helfen bei der Vorbereitung des Standortes, der Aufstellung des Geräts sowie bei der Schulung der Mitarbeiter.

HP 5898A
Bakterien-Identifizierungssystem

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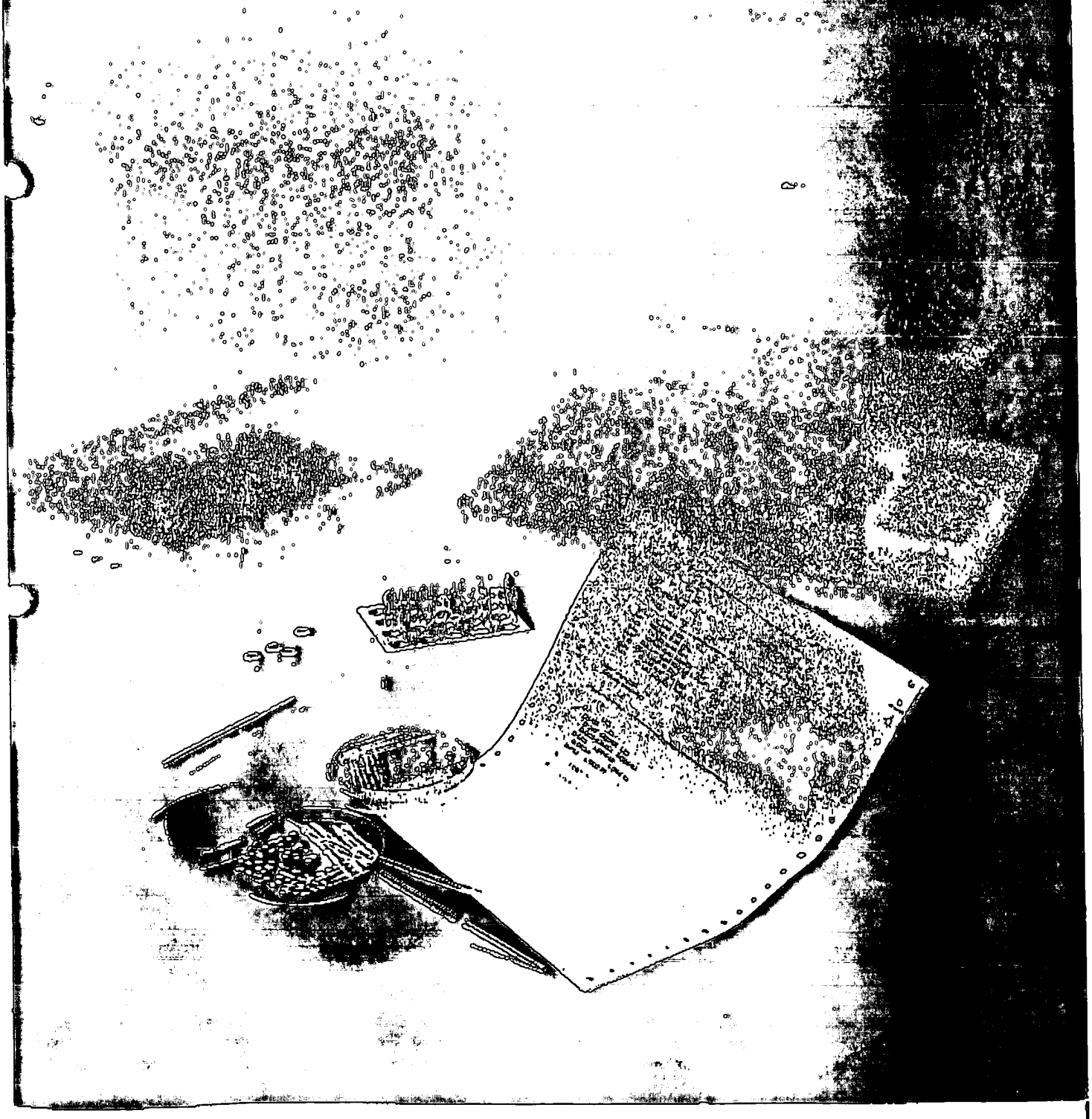
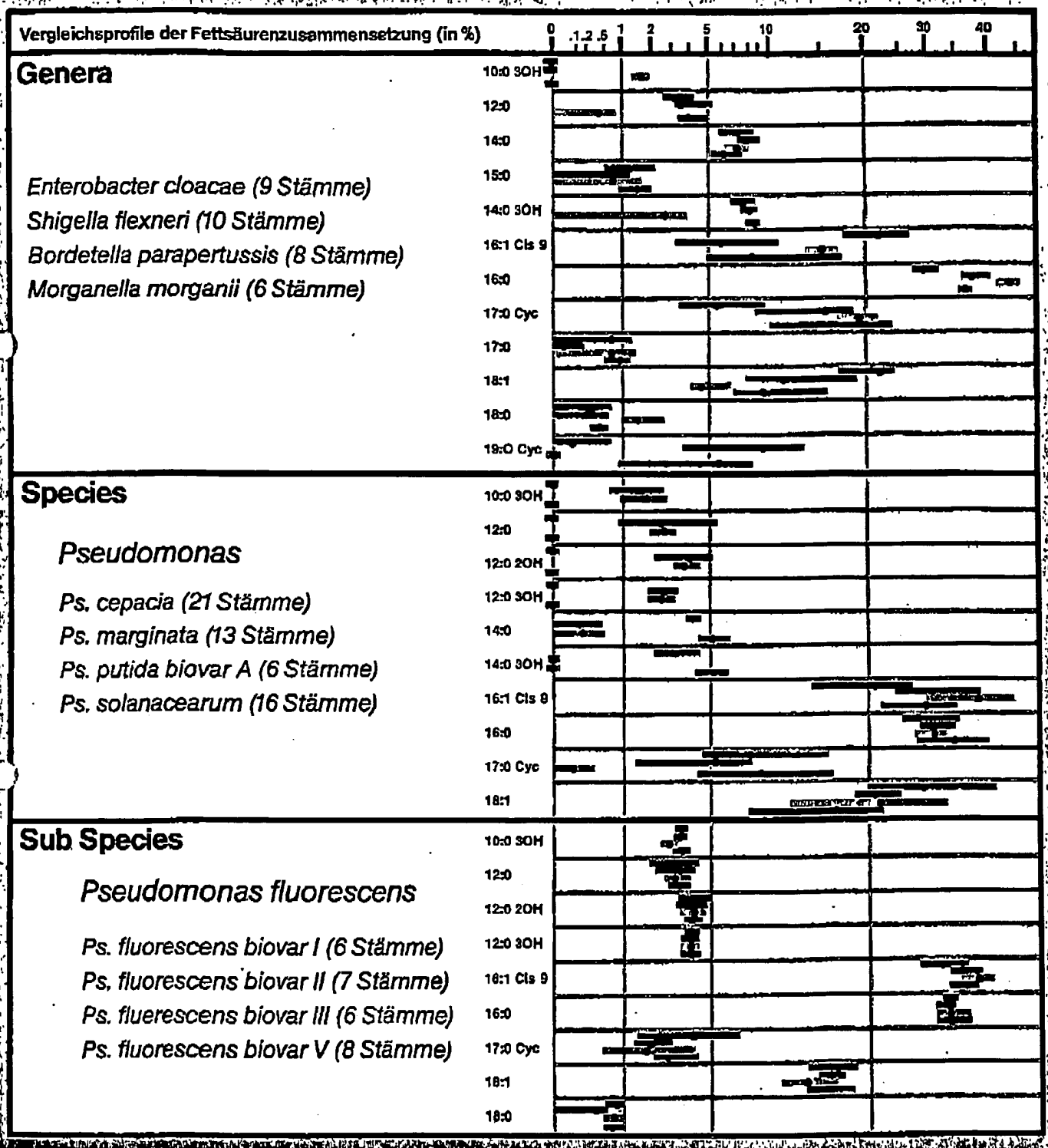


Das Fettsäureprofil korreliert mit der entsprechenden DNA-Struktur



Riboprinting

(Based on refs. 1, 2 and 21)

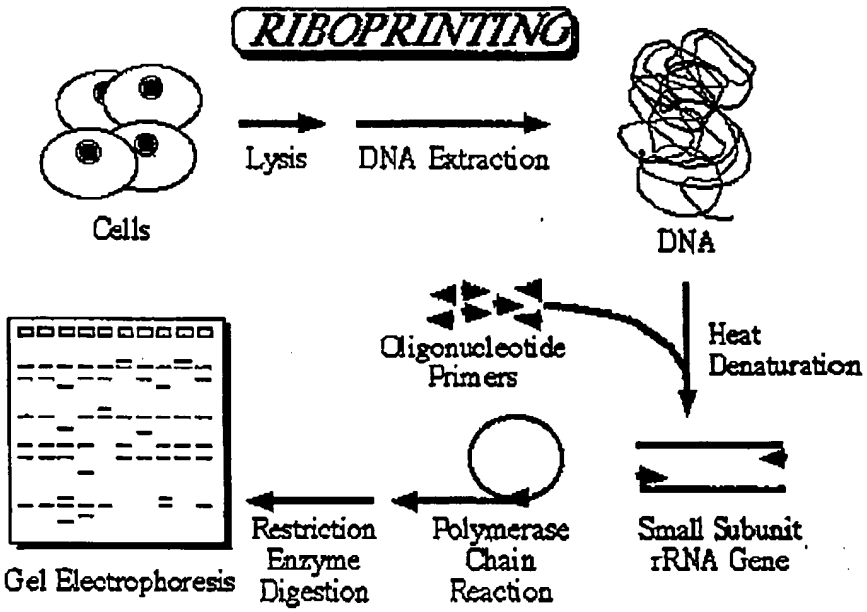
BACKGROUND

Many eukaryotic microorganisms have a simple structure that does not lend itself well to the traditional, morphology-based, classification systems. This is often due to secondary loss of cytoskeletal and flagellar microtubules which eliminates the very structures that are used most in protist classification schemes. Molecular methods can generate new characters for use in classification regardless of the morphological complexity of the organism of interest.

The polymerase chain reaction (PCR) amplifies large amounts of a specific DNA sequence from an organism using oligonucleotides complementary to the sequence to prime its replication by the DNA polymerase enzyme. As it theoretically can be used with a single cell as the starting material, this method clearly has many advantages where material is limiting. For a PCR-based method to generate useful data for classification, certain criteria must be met: it must not be dependent on prior knowledge of the genetic organisation of the organism; it must not be affected by the presence of bacterial DNA in the sample, as many eukaryotic microbes are found in association with numerous free-living or symbiotic bacteria; the DNA sequence to be analysed must be ubiquitous and conserved in the organisms of interest; the sequence must at the same time be variable enough so that species specific markers can be identified. Genes that match these apparently contradictory criteria include those encoding the small and large subunit ribosomal RNAs (rDNA).

The structure of rDNA is mosaic in nature, consisting of interspersed stretches of highly conserved, moderately conserved and divergent sequences. Regions can be identified that are conserved among all eukaryotic nuclear rDNAs but are distinct enough from bacterial rDNAs such that no amplification of the latter will occur in the PCR reaction. This method of specifically amplifying eukaryotic rDNA was first described by Medlin et al. [3] as a way to isolate these genes for cloning and DNA sequencing. This approach has been very successful and is largely responsible for the availability of hundreds of the eukaryotic small subunit rDNA sequences currently in the databases. However, DNA sequencing is a labour intensive and expensive proposition if many different related organisms are to be studied. Therefore a way of examining these genes is needed that is comparatively quick and inexpensive, so that multiple isolates of the same species as well as related species can be examined, but that will still yield useful data.

The method I call riboprinting combines the restriction site polymorphism analysis with the rDNA amplification method of Medlin to study sequence variation in the small subunit rDNA indirectly and it was first described by this name in 1991 [4]. The same approach was also developed independently by mycologists [5] and bacteriologists, where it is sometimes known as ARDRA (amplified ribosomal DNA restriction analysis; see [6]). I originally developed the method with the goal of studying relationships among species of *Entamoeba*, but the same approach has been used successfully in a number of taxonomically diverse protist and fungal genera [7-14].



The method is shown diagrammatically in above figure. DNA is isolated from the organisms and used as the template in a polymerase chain reaction. Two of the most highly conserved sequences in the small subunit rDNA are at each end of the small subunit gene. Oligonucleotide primers that are complementary to these regions can are used to amplify the small subunit rDNA. After the PCR is completed, a large quantity of what is essentially the complete small subunit rDNA has been generated. This amplified rDNA is then digested with restriction enzymes and the fragments separated in agarose gels along with a DNA size marker. The gels are then stained and photographed to provide a permanent record. Some of the restriction sites will fall in conserved regions and some in variable regions and this allows sequence polymorphisms to be detected as differences in the restriction fragment sizes.

As can be seen in the figure, species will often share one or more comigrating fragments for each restriction enzyme. Since the number of comigrating fragments will decrease as the gene sequences diverge, riboprinting can also be used to estimate genetic distances among species and algorithms have been developed that allow this to be done [15]. What riboprinting does is sample the gene sequence indirectly. The proportion of the gene sampled depends on the number of restriction enzymes used in the analysis, the size of the restriction enzyme recognition site, and how often they cut the gene. I routinely use 12 enzymes each with a four base recognition sequence and I find that this results in the sampling of 10-15% of the gene sequence without any cloning or sequencing of the DNA being necessary. In addition to generating estimates of genetic distance, the riboprint patterns can be used in parsimony analysis with each DNA fragment being viewed as an individual character.

Riboprinting is thus a method that does not require large amounts of starting material, it is quick, it is reproducible and it uses tools that can be found in almost every molecular biology laboratory.

METHODS

Polymerase Chain Reaction Amplification

1. Create a standard PCR master mix allowing 45 µl per reaction -

for 'Z' reactions thaw the solutions on ice and mix:

- 5xZ µl of 10xPCR buffer

- 0.5xZ µl of each primer in the pair (200 µM stock solution) (Note)
- 2xZ µl of each dNTP (10 µM stock)
- 0.5xZ µl of *Taq* Polymerase
- 30.5xZ µl of H_2O.

Briefly vortex the mix, spin it down in a microcentrifuge and place it on ice.

2. Place 5 µl of each DNA solution in 0.5 ml microcentrifuge tubes on ice, aliquot 45 µl of the master mix into each tube and overlay the reaction mixture with 50 µl of light mineral oil. (Note)

3. Program the thermal cycler to run 30 cycles of: 94° C - 1 minute; 55° C - 1.5 minutes; and 72° C - 2 minutes. Transfer the reaction tubes from the ice to the block and start the cycle sequence.

4. After cycling is over, extract the reactions with 100 µl of $CHCl_3$, briefly spin and remove the supernatant to a clean microcentrifuge tube. Success of the amplification reaction can be monitored by electrophoresis of an aliquot in a 0.8% agarose gel in 1X Tris Borate Buffer at 10 V/cm for 1 hour. The gel is then stained in 1 µg/ml ethidium bromide and destained in water for 15 minutes each on a rotary platform shaker before visualization of the DNA on a UV transilluminator and photographing the gel to obtain a permanent record.

5. Meanwhile, precipitate the amplification products by adding 0.1 volume of 3 M sodium acetate and 2 volumes of 100% ethanol. Vortex the mixture and let it stand for at least 5 minutes at room temperature. Spin the tube at full speed in a microcentrifuge for 15 minutes, wash the pellet with 200 µl of 70% ethanol by spinning for 5 minutes, then dry and resuspend it in 50 µl H_2O.

6. To visualize the full restriction pattern it is necessary to remove residual primers by passing the amplification product over a Sephacryl S400 (1 ml packed volume) spin column before digestion (see here for details).

7. The product is digested with restriction enzymes. The more enzymes used the more information is obtained - I recommend 12 enzymes all of which recognise a 4-base sequence.

CAVEATS

Riboprinting has its limitations:

1. Since only 10-15% of the gene sequence is sampled by this indirect method, significant variation can be present and will go undetected unless divergence falls within restriction sites. Similarly, if two restriction sites fall close together the resulting fragment will not be visible in the agarose gels and, if the sites are very close, the corresponding reduction in size of the adjacent restriction fragment may be too small to be noticed. Undoubtedly, riboprinting will miss some variation.

2. It is known that some species defined by reproductive isolation have small subunit rDNAs that are identical in sequence [16]. Therefore, identical riboprints do not indicate that the organisms necessarily belong to the same species.

3. Building trees based on fragment co-migration data assumes that all comigrating fragments are homologous. This will usually but not always be the case. Fragment co-migration can only be used to estimate relationships with any accuracy when the organisms are quite closely related [17]. Converting riboprints into restriction maps and using the number of shared restriction sites to

calculate genetic distances is known to provide more accurate estimates of relatedness.

4. Riboprinting is much less sensitive at detecting variation than isoenzyme analysis, for example. Isoenzyme variation is commonly seen among organisms that have identical riboprints. Restriction enzyme fingerprinting of less conserved genes may be informative in such cases.

5. If a mixture of small subunit rDNA sequence variants coexist in the same organism riboprinting may or may not reveal this fact. Where two forms of the gene exist differing at a single nucleotide position that coincides with a restriction site the resulting pattern will likely be interpreted as resulting from incomplete restriction enzyme digestion of the PCR product even if both variants make up a significant proportion of the gene complement. One such case has been identified, but only because a complete gene sequence was already available [18]. Minor variants may not be noticed at all.

6. Occasionally, related species will differ by the presence of an intron in the small subunit rDNA of one but not the other [19]. Such a situation will be easily recognized as the small subunit rDNA amplification products will differ in size by several hundred basepairs. The presence of introns, while interesting, makes comparison of riboprint patterns for phylogenetic analysis difficult at best unless they are present in in all isolates being compared.

7. Mixtures of organisms will produce difficulty in interpretation of restriction digest patterns. In some cases their existence will be obvious from the presence of two or more bands in the undigested product gel patterns. However, this will not always be the case. Mixtures will give rise to patterns where the sum of the fragment sizes exceeds the size of the amplification product.

As long as these limitations are recognized, riboprinting is a fast, reproducible way to obtain useful data from small numbers of organisms. It has the ability to detect cryptic genetic variation, to identify morphologically conservative organisms, to uncover misidentified organisms and culture mix-ups, to act as an independent check on DNA sequencing accuracy, and to provide data that can be used to estimate relatedness and to generate phylogenetic trees.

Note 1: The choice of the primer pair used depends on the species involved. Amplification of the SSU-rDNA of many species uses the primer pair RD5 and RD3 which amplifies almost the entire SSU-rRNA gene. Amplification of the SSU-rDNA of trichomonads uses the primer pair TRD5 and TRD3 [20], while diplomonads and microsporidia would also require group specific primers due to sequence divergence in the primer binding regions of the genes. Identification of all species then relies on digestion of the amplification product with a battery of restriction enzymes. (Back)

Note 2: It is often useful to try at least two DNA concentrations for each DNA preparation, eg. a 1:10 and a 1:100 dilution. Include a negative control using 5 µl H_2O instead of DNA. (Back)

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Friulimicins: Novel Lipopeptide Antibiotics with Peptidoglycan Synthesis Inhibiting Activity from *Actinoplanes friuliensis* sp. nov.

I. Taxonomic Studies of the Producing Microorganism and Fermentation

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A strain that produces new lipopeptide antibiotics is a new species of the genus *Actinoplanes* for which we propose the name *Actinoplanes friuliensis* (type strain: HAG 010964). The strain is an actinoplanete actinomycete having cell wall II composition and forming sporangia. Comparisons with *Actinoplanes* spp. which have similarities with our isolate, including fatty acid analysis, showed that the isolate belongs to a new species. Taxonomic studies and fermentation are presented.

The genus *Actinoplanes* is one of the most important genera among actinomycetes in the production of secondary metabolites. Gardimycin[1] and teicoplanin[2] are two reported antibiotics from the genus *Actinoplanes*. Lipopeptides have been reported from *Actinoplanes nipponensis*[3]. The α-glucosidase inhibitor acarbose is similarly a product of *Actinoplanes* sp.[4].

In our screening program for new antibiotics active against methicillin-resistant *Staphylococcus aureus*, a strain that produced a group of new lipopeptide antibiotics (the structure elucidation will be presented in the following paper) was isolated from a soil sample collected in northern Italy in the region of Friuli. This strain contained *meso*-diaminopimelic acid in its cell wall and xylose was found as characteristic sugar in whole cell hydrolysates. Thus it is a member of the family *Micromonosporaceae* according to the taxonomic proposal of STACKEBRANDT *et al.*[5].

The colony is typically orange and forms globose sporangia, and so it belongs to the genus *Actinoplanes*. Studies of similar *Actinoplanes* species using the methods of the International Streptomycetes Project[6] and chemotaxonomic methods lead us to conclude that our strain is a member of a new species we call *Actinoplanes friuliensis* sp. nov. The strain has been deposited at the German Culture Collection (DSMZ) under number DSM 7358.

Materials and Methods

Isolation

Strain HAG 010964 was isolated from a soil sample collected at the garden entrance of a house in the Friuli Province, Italy on June 3, 1987, using the chemotactic method of PALLERONI[7] and starch-casein-sulfate agar medium recommended by VOBIS[8].

Bacterial Strains

The strains used in this study are shown in Table 1. Other strains than the type strains were *Actinoplanes utahensis* FH 2264 and our new lipopeptide producing strain HAG 010964.

For the detection of the most similar strains to the lipopetide producer the following strains were investgated by their morphological characters like colony formation and shape of sporangia on ISP media: *Actinoplanes auranticolor* ATCC 15330, *A. brasiliensis* ATCC 25844, *A. campanulatus* IMET 9244, *A. consettensis* ATCC 49799,

Table 1. Strains of the genus *Actinoplanes* used for direct comparative studies.

Species	Strain No.	Source
Actinoplanes brasiliensis Thiemann et. al. 1969	FH 2237	ATCC 25844^T
"Actinoplanes nipponensis" Routien 1977 [1]	FH 2241	ATCC 31145^T
Actinoplanes utahensis Couch 1963	FH 2264	NRRL 12052
Actinoplanes friuliensis sp. nov.	HAG 010964	own isolate

^{T} type strain of the species; [1] the species *"A. nipponensis"* has not been validly published according to Rule 27 of the International Code of Nomenclature of Bacteria (Lapage *et al.*, 1992 [21])

A. cyaneus ATCC 21983, *A. deccanensis* ATCC 21983, *A. derwentensis* ATCC 49798, *A. digitatis* ATCC 15349, *A. durhamensis* ATCC 49800, *A. ferrugineus* ATCC 29868, *A. garbadiensis* FH 2243, *A. globisporus* ATCC 23056, *A. humidus* ATCC 49801, *A. ianthinogenes* ATCC 27366, *A. italicus* IFO 13661, *A. kinshahensis* IFO 13997, *A. liguriae* ATCC 31048, *A. lobatus* ATCC 15350, *A. missouriensis* ATCC 14538, *A. nipponensis* ATCC 31145, *A. palleroni* IFO 14961, *A. philippinensis* ATCC 12427, *A. rectilineatus* ATCC 29234, *A. regularis* ATCC 31417, *A. teichomyceticus* ATCC 31121, *A. utahensis* DSM 43147 and NRRL 12052. This date will be presented separately.

Morphology and Physiology

The morphological and physiological characteristics of the strains were observed by using agar cultures on the various media described by SHIRLING and GOTTLIEB[6]: yeast extract-malt extract agar (ISP 2), oatmeal agar (ISP 3), inorganic salt-starch agar (ISP 4), glycerol-asparagine agar (ISP 5), peptone-yeast extract iron agar (ISP 6) and tyrosine agar (ISP 7), incubated for 10 days at 28°C. For scanning electron microscopy the strain was grown on ISP 3 agar. The small agar piece was prepared using the method of WINK *et al.*[9].

Utilization of carbohydrates was investigated on ISP 9 medium (SHIRLING and GOTTLIEB[6]) using a microtiter plate technique with twelve well plates. Sodium chloride tolerance was tested on microtiter plates (six-well) too using a technique based on the method of KUTZNER[10]. A fingerprint of enzymatic activities was obtained with the help of API 20E and API ZYM test strips[11~13].

Antimicrobial Spectrum

For the antimicrobial spectrum we used the strains described by GOODFELLOW in BERGEY's manual[14]. The bacteria were grown on Mueller Hinton agar and the fungi on Czapek Dox. For metabolite production the *Actinoplanes* strains were incubated in a medium containing soluble starch (10.0 g/liter), yeast extract (2.0 g/liter), glucose (10.0 g/liter), glycerol (10.0 g/liter), cornsteep liquor (2.5 g/liter), peptone (2.0 g/liter), NaCl (1.0 g/liter) and $CaCO_3$ (3.0 g/liter) for five days in a shaking flask culture at 28°C. After cultivation the whole culture was extracted with methanol, evaporated and dissolved in water.

Chemotaxonomic Analysis

Analysis of the whole-cell diaminopimelic acid isomers and the sugars was done by the method of HASEGAWA *et al.*[15]. The phospholipids were analyzed by the method of KUTZNER *et al.*[10].

For analysis of whole-cell fatty acid composition we developed a rapid method based on the method of MÜLLER *et al.*[16] and our own database. The strain was incubated in ISP 2 broth for 7 days at 28°C and 1 ml was transferred to the center of a sterile Sartorius filter (SM 11106) placed on an agar medium containing starch (10 g/liter), glucose (10 g/liter), glycerol (10 g/liter), cornsteep liquor (2.5 g/liter), peptone (5 g/liter), yeast extract (2 g/liter), NaCl (1 g/liter) and $CaCO_3$ (3 g/liter). Incubation was carried out for 5 days at 28°C. Two loops of cell material were transferred into 10 µl of distilled water in a microtube, 35 µl of methanolic TMSH were added and the sample was mixed. The sample was dried with a nitrogen stream (Barkey Evaporator) and extracted into 100 µl of a mixture of 9 volumes of *tert*-butyl

methyl ether and *n*-hexane (1 : 1, v/v) and one volume of methanol. The extract was used directly for the GC analysis, which was done with an HP 6890 GC (Hewlett-Packard).

Fermentation

The friulimicins were produced by fermentation in a 50-liter Braun Fermentation stainless steel stirred vessel. Frozen vegetative mycelium of *Actinoplanes friuliensis* was used at 1% to inoculate 100 ml of seed medium in a 300 ml Erlenmeyer flask. The seed medium consisted of sucrose 30 g/liter, KNO_3 2 g/liter, K_2HPO_4 1 g/liter, $MgSO_4 \cdot 7H_2O$ 0.5 g/liter, KCl 0.5 g/liter, $FeSO_4 \cdot 7H_2O$ 0.01 g/liter, yeast extract 2 g/liter and peptone 5 g/liter. The seed flasks were incubated for 120 hours at 30°C on a rotary shaker at 180 rpm. The fermenter was charged with 40 liters of a medium consisting of sucrose 11 g/liter, meat extract 6 g/liter, yeast extract 0.3 g/liter, $MgSO_4 \cdot 7H_2O$ 0.6 g/liter, K_2HPO_4 0.1 g/liter, $FeCl_3 \cdot 6H_2O$ 10 μmol and L-valine 0.6 g/liter. The medium-resin mixture was sterilized in the fermenter at 121°C for 1 hour. The fermenter was inoculated at 1% with the seed flask growth. During fermentation the temperature was controlled at 28°C. The stirring rate was 100 rpm and the air flow rate was 1 v/v/minute. Antifoam (Desmophen) was initially at 0.01%. The fermentation was terminated at 120 hours.

Fermentation Analysis

The fermentation was monitored on-line for changes in pH. The fermenter was sampled daily to evaluate growth and product formation. The formation of the compound was monitored with an HPLC system. The column used was a Nucleosil 120 RP 18 (120×4.6 mm with a 20×4.6 mm precolumn). A gradient with a potassium phosphate buffer (pH 7.0, 10 mM) and acetonitrile was used. The flow rate was between 1.5 and 2 ml/minute. With this system crude mixtures of the compounds could be detected in the culture filtrate. The retention time was between 10 and 20 minutes.

Results

Characteristics of Strain HAG 010964

Vegetative mycelium developed well on the ISP media tested (Table 2). Aerial mycelium was not formed and a red soluble pigment was produced on medium ISP 7. After 10 days on ISP 3, sporangia were formed which show an irregular shape with a smooth surface in the scanning electron micrograph. The surface of the spores was smooth.

The vegetative mycelium was orange on all the ISP media used. All carbohydrates which were tested could be utilized by the strain HAG 010964 (Table 3). The enzymatic activities are shown in Table 4. Apart from lipase (C14) and α-fucosidase, all reactions with API ZYM were positive, and with API 20E no activities were detected for H_2S production, tryptophan deaminase and indole production. Antibacterial activity (Table 5) was detectable only with the starch medium, which is also described as a production medium.

Comparison of strain HAG 010964 with *Actinoplanes brasiliensis* FH 2237 (ATCC 25844), "*A. nipponensis*" FH 2241 (ATCC 31145) and *A. utahensis* FH 2264 (NRRL 12052). After the characterization of all the *Actinoplanes* species which are listed under the point materials and methods the strains *Actinoplanes brasiliensis* FH 2237, "*A. nipponensis*" FH 2241 and *A. utahensis* FH 2264 show most similarities to the strain HAG 010964 basing on data of colony morphology, pigmentation and shape of sporangia, so we used this strains for the comparing studies. Between *A. utahensis* the strain FH 2264 shows more similarity to HAG 010964 than the type strain DSM 43147 and therefore we used FH 2264. The strain *A. brasiliensis* FH 2237 showed yellow-orange substrate mycelium, while the mycelium of the other strains was orange. Also *A. brasiliensis* FH 2237 is the only strain which produceed sparse white mycelium on medium ISP 3. All four strains produced red soluble pigment on ISP 7 but only "*A. nipponensis*" FH 2241 also produced brown one on ISP 6. HAG 010964 and *A. utahensis* FH 2264 utilized all carbohydrates. *A. brasiliensis* FH 2237 showed inferior utilization of xylose, inositol and raffinose, and "*A. nipponensis*" FH 2241 did not utilize xylose, mannitol, fructose and raffinose.

With regard to enzymatic activities, API ZYM and API 20E reacted much more positively with HAG 010964 than with the three other strains. In the API 20E pattern *A. brasiliensis* FH 2237, "*A. nipponensis*" FH 2242 and *A. utahensis* FH 2264 looked very similar and only acetoin production and gelatinase activity could be found in all four strains. *A. brasiliensis* FH 2237 and *A. utahensis* FH 2264 showed no antibacterial activity on any of the tested media. Antibacterial activity against *Bacillus subtilis* was found on all four media for the strain "*A. nipponensis*" FH 2242. Strain HAG 010964 showed activity against *Staphylococcus aureus*, *Micrococcus luteus* and *Bacillus subtilis* only on starch-containing production medium. In the sporangial morphology observed with the scanning electron microscope, strain HAG 010964 showed the most irregular shapes. The sporangia of *A. brasiliensis* FH 2237 are much

Table 2. Colonial characteristics of *Actinoplanes brasiliensis* FH 2237, "*A. nipponensis*" FH 2241, *A. utahensis* FH 2264 and *A. friuliensis* sp. nov. HAG 010964.

Culture medium	Strains FH 2237	FH 2241	FH 2264	HAG 010964
ISP 2	SM yellow-orange AM none SP none	SM orange AM none SP none	SM orange AM none SP none	SM orange AM none SP none
ISP 3	SM yellow-orange AM white SP none	SM orange AM none SP none	SM orange AM none SP none	SM orange AM none SP none
ISP 4	SM yellow-orange AM none SP none	SM orange AM none SP none	SM orange AM none SP none	SM orange AM none SP none
ISP 5	SM yellow-orange AM none SP none	SM orange AM none SP none	SM orange AM none SP none	SM orange AM none SP none
ISP 6	SM yellow-orange AM none SP none	SM orange AM none SP brown	SM orange AM none SP none	SM orange AM none SP none
ISP 7	SM yellow-orange AM none SP red	SM orange AM none SP red	SM orange AM none SP red	SM orange AM none SP red

Formation and color of: SM, substrate mycelium; AM, aerial mycelium; SP, soluble exopigment

Table 3. Utilization of carbohydrates by *Actinoplanes brasiliensis* FH 2237, "*A. nipponensis*" FH 2241, *A. utahensis* FH 2264 and *A. friuliensis* sp. nov. HAG 010964.

Carbohydrate	Strains FH 2237	FH 2241	FH 2264	HAG 010964
Glucose	+	+	+	+
Arabinose	+	+	+	+
Sucrose	+	+	+	+
Xylose	(+)	-	+	+
Inositol	(+)	(+)	+	+
Mannitol	+	-	+	+
Fructose	+	-	+	+
Rhamnose	+	+	+	+
Raffinose	(+)	-	+	+

-, growth no better than the negative control (basal medium with water);
(+), growth better than the negative control but not as good as the positive control;
+, growth as good as the positive control (basal medium with glucose).

Table 4. Enzymatic activities of *Actinoplanes brasiliensis* FH 2237, "*A. nipponensis*" FH 2241, *A. utahensis* FH 2264 and *A. friuliensis* sp. nov. HAG 010964.

Physiological parameter	Strains FH 2237	FH 2241	FH 2264	HAG 010964
API ZYM				
Alkaline phosphatase	+	+	+	+
Esterase (C 4)	+	+	+	+
Esterase (C 8)	-	+	+	+
Lipase (C 14)	+	-	-	-
Leucine arylamidase	-	+	+	+
Valine arylamidase	-	-	-	+
Cystine arylamidase	+	-	-	+
Trypsin	+	-	-	+
Chymotrypsin	+	-	+	+
Phosphatase acid	+	+	+	+
Naphthol-AS-BI-phosphohydrolase	+	-	+	+
α-Galactosidase	+	-	+	+
β-Galactosidase	-	-	+	+
β-Glucuronidase	+	-	-	+
α-Glucosidase	+	+	+	+
β-Glucosidase	+	+	+	+
N-Acetyl-β-glucoseamidase	+	+	+	+
α-Mannosidase	+	-	-	+
α-Fucosidase	-	-	-	-
API 20E				
β-Galactosidase	-	-	-	+
Arginine dihydrolase	-	-	-	+
Lysine decarboxylase	-	-	-	+
Ornithine decarboxylase	-	-	-	+
Citrate utilization	+	-	-	+
H_2S production	-	-	-	-
Urease	-	-	-	+
Tryptophan deaminase	-	-	-	-
Indole production	-	-	-	-
Acetoin production	+	+	+	+
Gelatinase	+	+	+	+

more compact and regular and similar to the sporangia of *A. utahensis* FH 2264 as shown in Fig. 1. For "*A. nipponensis*" FH 2242, production of sporangia was undetectable. In all four *Actinoplanes* strains *meso*-diaminopimelic acid was found in whole cell extracts as well as xylose as characteristic sugar. The phospholipide type of all strains was PII containing phosphatidyl ethanolamine. Main fatty acids of all four strains were 15:0 Iso, 15:0 Anteiso, 16:0 Iso, 17:0 Anteiso, 17:1 Cis 9, 17:0, 18:1 Cis 9 and 18:0.

Fermentation

Beside a suitable C-source and phosphate the wild type strain needs a N-source like meat extract or soya peptone and magnesium ions seems to be necessary for a good production of the lipopeptides. In contrast to magnesium calcium salts at 10 mM decreased the lipopeptide production to 15%. The strain does not exhibit a strong phosphate or ammonia inhibition. Amino acids like L-valine, L-leucine and L-isoleucine or the corresponding keto-acids or acids, which are precursor substances of the fatty acid biosynthesis, lead to an increased production of the desired components of the lipopeptide complex (see

Table 5. Antimicrobial activities from *Actinoplanes brasiliensis* FH 2237, "*A. nipponensis*" FH 2241, *A. utahensis* FH 2264 and *A. friuliensis* sp. nov. HAG 010964.

Culture broth	*Actinoplanes brasiliensis* FH 2237				*Actinoplanes nipponensis* FH 2241			
	Soy meal	ISP 2	Starch	ISP 3	Soy meal	ISP 2	Starch	ISP 3
Staphylococcus aureus	0	0	0	0	0	11	0	0
Escherichia coli	0	0	0	0	0	0	0	0
Micrococcus luteus	0	0	0	0	0	0	0	0
Pseudomonas aeruginosa	0	0	0	0	0	0	0	0
Streptomyces murinus	0	0	0	0	0	0	0	0
Bacillus subtilis	0	0	0	0	21	21	15	15
Candida albicans	0	0	0	0	0	0	0	0
Saccharomyces cerevisiae	0	0	0	0	0	0	0	0
Aspergillus niger	0	0	0	0	0	0	0	0
Culture broth	*Actinoplanes utahensis* FH 2264				*Actinoplanes friuliensis* HAG 010964			
	Soy meal	ISP 2	Starch	ISP 3	Soy meal	ISP 2	Starch	ISP 3
Staphylococcus aureus	0	0	0	0	0	0	16	0
Escherichia coli	0	0	0	0	0	0	0	0
Micrococcus luteus	0	0	0	0	0	0	15	0
Pseudomonas aeruginosa	0	0	0	0	0	0	0	0
Streptomyces murinus	0	0	0	0	0	0	0	0
Bacillus subtilis	0	0	0	0	0	0	16	0
Candida albicans	0	0	0	0	0	0	0	8
Saccharomyces cerevisiae	0	0	0	0	0	0	0	0
Aspergillus niger	0	0	0	0	0	0	0	0

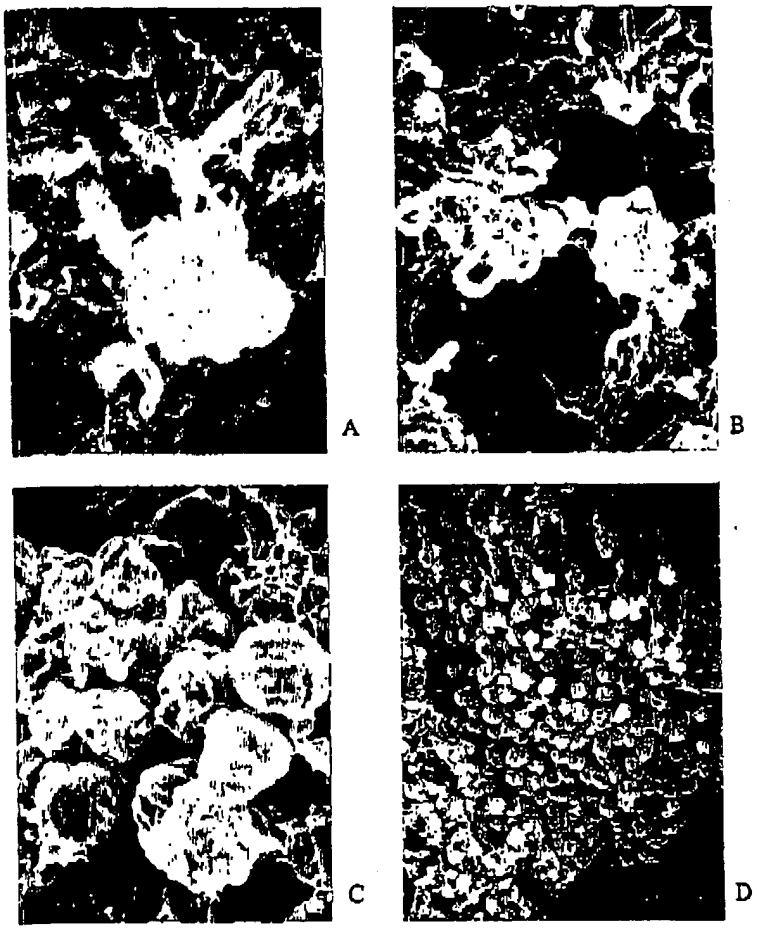
Table 7). Using L-valine as a precursor the compounds B and D are exclusively produced. Using L-leucine the compounds A and C and by using L-isoleucine the compounds E, F, G and H are produced. With this results it is possible to produce the compounds B and D in a yield of 1.5 g/liter in the medium which has been described above. The time course of the production of this two compounds of the friulimicin/amphomycin complex is shown in Fig 2.

Discussion

On the base of chemotaxonomic and morphological properties, strain HAG 010964 can be classified as a member of the genus *Actinoplanes*. Strains of the genus *Actinoplanes* present cell wall chemotype II according to the classification of LECHEVALLIER and LECHEVALLIER[17], with *meso*- and/or 3-hydroxydiaminopimelic acid and glycine, in combination with xylose and arabinose as characteristic sugar in the hydrolysate of whole organisms, and glycolyl type according to UCHIDA and AIDA[18]; phospholipid type PII according to the classification of LECHEVALLIER[19]; fatty acid type 2 c with characteristic *iso*- and *anteiso*-branched fatty acids according to KROPPENSTEDT[20]. Mycolic acid is absent. In strain HAG 010964 we found *meso*-diaminopimelic acid and xylose. The phospholipid type of the strain is PII and the *iso*- and *anteiso*-branched fatty acids are characteristic. A significant cultural marker is the orange substrate mycelium and the absence of aerial mycelium, combined with the production of sporangia on the surface of the colony. Each sporangium contains numerous globose to subglobose spores, which become flagellated in aqueous habitats. In addition the sporangial development which could be studied in SEM showed the branching and septa formation before spore formation indicating affiliation to the genus *Actinoplanes* (VOBIS[8]). The irregular arrangement of the spore chains excludes the former *Ampullariella* species, which have strict parallel rows of spore chains and distinct rod-shaped sporangiospores. Thus only "*A. nipponensis*", *A.*

Fig. 1. Scanning electron micrograph of sporangia formation of the different *Actinoplanes* species.

A) strain HAG 010964 grown on ISP 3 agar for 10 days at 28°C (magnification ×10,000).
B) strain HAG 010964 grown on ISP 3 agar for 14 days at 28°C (magnification ×5,000).
C) strain *A. brasiliensis* FH 2237 grown on ISP 3 agar for 14 days at 28°C (magnification ×5,000).
D) strain *A. utahensis* FH 2264 grown on ISP 3 agar for 14 days at 28°C (magnification ×1,500).



brasiliensis and *A. utahensis* showed very similar colony morphology and pigmentation. In carbohydrate utilization and enzymatic activities strain HAG 010964 shows a lot of differences to *A. brasiliensis* FH 2237, "*A. nipponensis*" FH 2241 and *A. utahensis* FH 2264.

In this group of species closely related to HAG 010964 the species *A. utahensis* and *A. brasiliensis* form sporangia with a regular globose shape which is different from that of HAG 010964. In difference to *A. brasiliensis*, *A. nipponensis* and *A. utahensis* the strain HAG 010964 contain 15:1 IsoG and 16:1 IsoG as typical fatty acids but has no 16:0 acid. All these studies and results as well as the selective fermentation of new lipopetide compounds which have not been described from *Actinoplanes* lead us to conclude, that HAG 010964 is a new species, which we named *Actinoplanes friuliensis* sp.nov.

Description of *Actinoplanes friuliensis* sp. nov.: Produces orange to yellow orange substrate mycelium on the different ISP media. Aerial mycelium is not formed. A red soluble pigment is only produced on medium ISP 7. Melanoid pigment is not produced. Glucose, arabinose, sucrose, xylose, inositol, mannitol, fructose, rhamnose and raffinose could be utilized. Sporangia are formed on the different ISP media. The shape of sporangia is irregular to globose. Cells contain *meso*-diaminopimelic acid and xylose. Characteristic phospholipid is phosphatidyl

Table 6. Fatty acid pattern of *Actinoplanes brasiliensis* FH 2237, "*A. nipponensis*" FH 2241, *A. utahensis* FH 2264 and *A. friuliensis* sp. nov. HAG 010964.

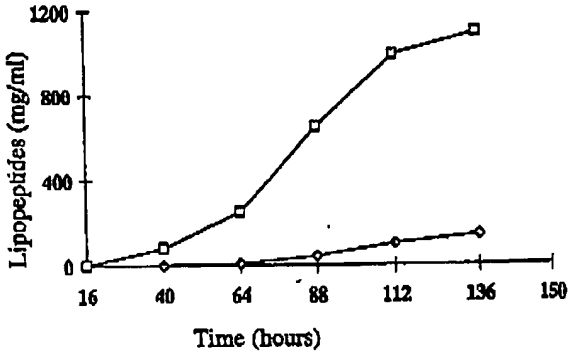
Fatty Acid	Strains FH 2237	FH 2241	FH 2264	HAG 010964
14 : 0 Iso	3.0	4.0	-	2.7
15 : 1 Iso G	-	-	-	5.5
15 : 0 Iso	13.0	9.5	3.6	15.4
15 : 0 Anteiso	18.5	15.5	1.0	12.0
16 : 1 Iso G	-	-	-	3.5
16 : 0 Iso	17.3	21.4	8.0	14.0
16 : 1 Cis 9	3.0	2.2	27.0	4.5
16 : 0	5.0	7.2	22.0	-
17 : 0 Iso	3.4	1.2	6.0	1.8
17 : 0 Anteiso	8.9	8.0	3.0	5.0
17 : 1 Cis 9	4.9	3.0	6.0	10.0
17 : 0	9.4	6.0	3.0	4.5
18 : 1 Cis 9	5.0	9.0	8.0	8.2
18 : 0	6.0	10.0	3.0	9.5

Table 7. Lipopeptides from *Actinoplanes friuliensis* sp. nov. HAG 010964.

Compound A	Amphomycin Type
Compound B	Amphomycin Type
Compound C	Friulimycin A
Compound D	Friulimycin B
Compound E	Amphomycin Type
Compound F	Friulimycin C
Compound G	Amphomycin Type
Compound H	Friulimycin D

Fig. 2. Time course of a selective fermentation in a 20-liter fermenter with additional 10 mmol Val.

-□- peptide B and -◇- peptide D.



ethanolamine. Fatty acids are 15 : 1 IsoG, 15 : 0 Iso, 15 : 0 Anteiso, 16 : 0 Iso, 17 : 0 Iso, 17 : 0 Anteiso, 17 : 1 Cis 9, 17 : 0, 18 : 1 Cis 9 and 18 : 0. Type strain is HAG 010964 (DSM 7358), a antibacterial lipopeptides producing strain.

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Automated System Rapidly Identifies and Characterizes Microorganisms in Food

Working from an isolated bacterial colony, the system performs all the process steps required to characterize bacteria to the strain level

JAMES BRUCE

PATHOGENIC AND SPOILAGE MICROORGANISMS IN FOOD processing environments can cause costly and time-consuming delays in production and processing. Certain contaminants, such as *Salmonella*, *Listeria monocytogenes*, *Escherichia coli*, or *Staphylococcus aureus*, can result in complete shutdown of food production until the source of contamination is identified and eliminated. A premature shutdown based on incomplete or presumptive information may result in lost production; conversely, a delay in pathogen identification may result in an even more costly product recall. Management should not be placed in a position of making a high-risk, high-stakes guess. Meaningful, reliable, detailed information is required. Time and accuracy are of the essence.

Standard microbiological techniques, however, can take several days to arrive at a species identification. Traditional methods for bacterial isolation and identification at the species level are based on secondary characteristics of the bacteria and can be tedious and time-consuming. These traditional methods may require multiple growth conditions and several days of biochemical testing to arrive at a species identification. Once a conventional analysis has been completed, the results do not necessarily provide the resolution required to identify the source of contamination or infection. Since different strains of the same species exhibit very similar biochemical characteristics, but can often exhibit different degrees of virulence, it is often necessary to continue with a more detailed analysis, such as serological tests for more precise characterization. Such testing can lengthen the investigation process by several days to several weeks an unacceptably long time when the business stakes are so high.

During the early 1980s, a variety of molecular techniques were developed at research laboratories for DNA analysis and gene exploration. In the mid-1980s some of these technologies were applied to bacterial analysis. One technique, called ribotyping, evolved for characterization of organisms using restriction fragments of nucleic acids from bacterial genomes (Webster, 1983, 1988; Grimont and Grimont, 1986; Jacquet et al., 1992). By hybridizing a ribosomal probe to size-separated DNA restriction fragments, this method can permit accurate and reliable characterization and identification of bacteria.

Although this technology has been used widely in research laboratories, it depends on highly skilled practitioners to achieve meaningful and reliable results. In addition, many investigators have applied this technique to meet their very specific needs, reducing the ability to compare results between laboratories. To address these and other important issues in microbiological characterization, the Food Quality Management Systems team at the DuPont Company developed a fully automated ribotyping system, the RiboPrinter Microbial Characterization System (Fig. 1).

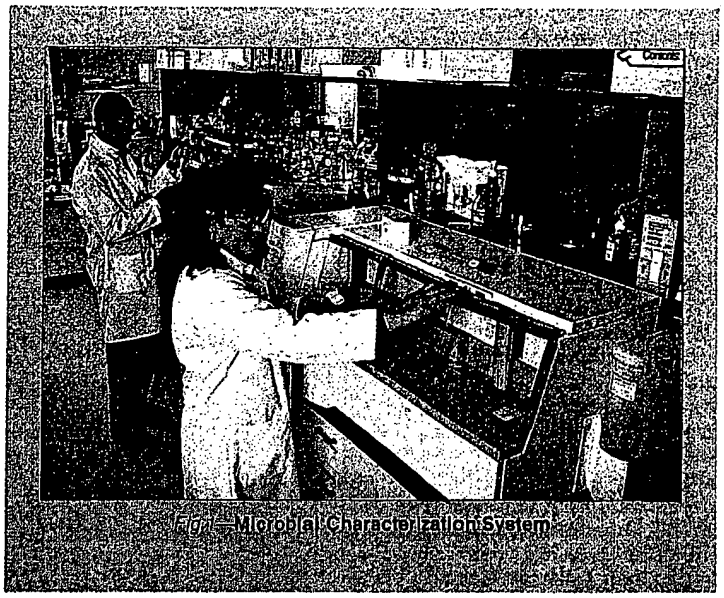

Fig. 1—Microbial Characterization System

Automated Ribotyping

The system combines many molecular processing steps in a stand-alone, automated ribotyping instrument designed specifically to satisfy the needs of the food industry. Working from an isolated bacterial colony, the system performs all the process steps required to characterize the bac-

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teria to the strain level, from cell lysis to image analysis. When DNA fragments from different bacterial strains, cut with *Eco*RI restriction enzyme, are size-separated and hybridized with a labeled rRNA operon probe, each strain produces a unique fragment pattern. From this fragment pattern data, the system uses a series of proprietary algorithms to generate a RiboPrint pattern, then characterizes, archives, and compares these patterns to a supplied database. This comparison can result in the identification of the organisms of interest at a genus, species, and strain level. The automated system also compares the pattern for each new sample against all the other patterns run on the system to determine similarity. This process allows the system to characterize samples as alike or different even when the tested strain is not part of the identification database. This characterization function is especially useful when investigating new or unusual bacteria.

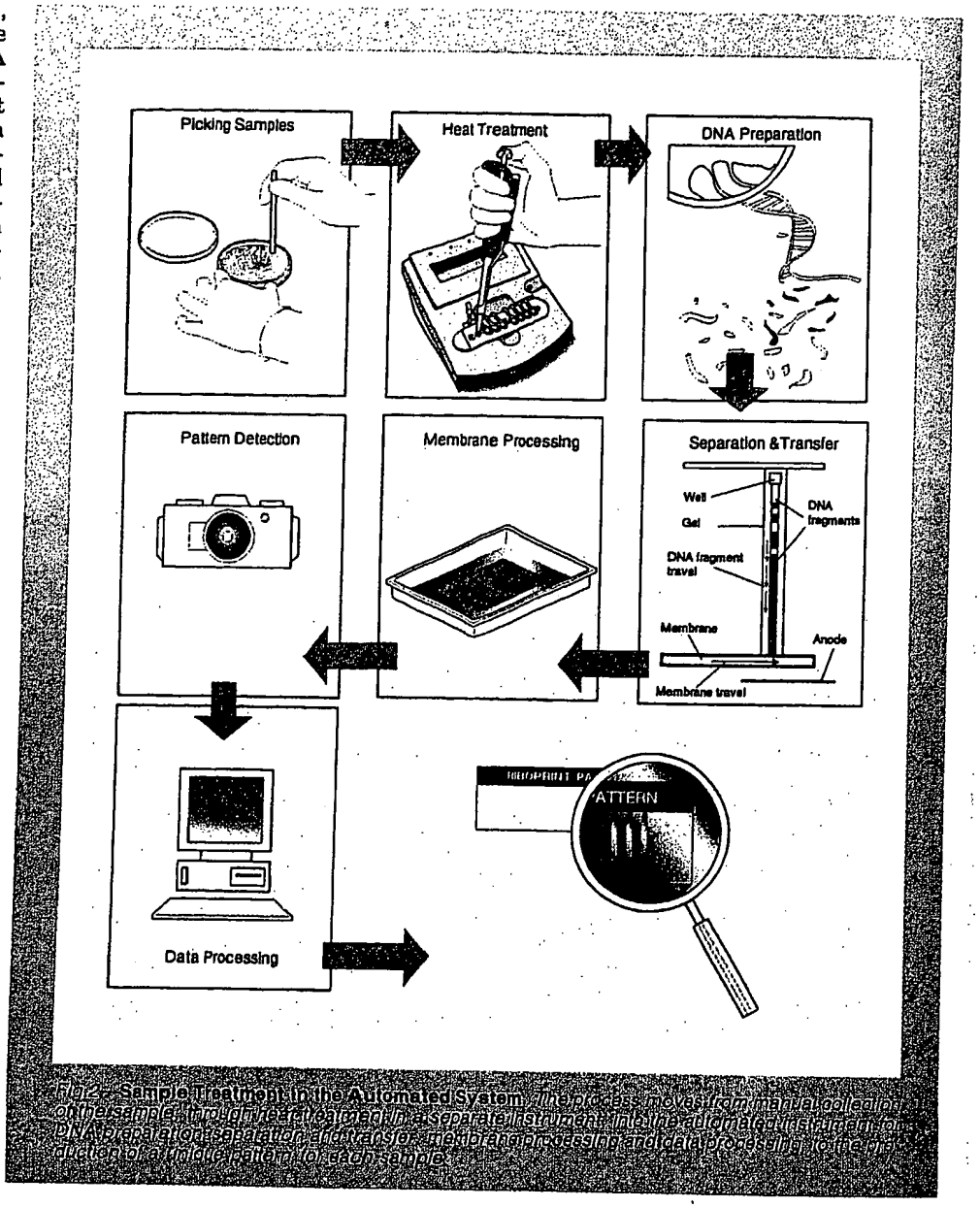

Fig. 2. Sample Treatment in the Automated System. The process moves from manual collection of the sample, through heat treatment in a separate instrument, into the automated instrument for DNA preparation, separation and transfer, membrane processing and data processing, to the production of a riboprint pattern for each sample.

Because of the importance of accuracy and time in characterizing, identifying and eventually eliminating undesirable organisms for the food industry, this automated system has been designed to reduce the time involved in each step, while eliminating the uncertainties of classical strain characterization/identification. The result is a process that requires 8 hr to produce reliable and accurate results from isolated colonies.

How the System Works

Fig. 2 illustrates the process used to prepare and analyze microbiological samples. The following discussion summarizes the steps involved in obtaining a pattern for a microorganism under investigation.

Engineering advances in the equipment and specially manufactured reagents and carriers make it possible to achieve extreme precision in volume and concentration of sample and reagents, yielding a high confidence, reproducible process.

• **Growth and Collection of Cultures.** Isolated bacterial strains are inoculated and grown overnight on brain heart infusion (BHI) agar plates. Bacterial samples are obtained using a simple colony pick and then resuspended in buffer in a micro-centrifuge tube. The diluted sample is transferred into a special sample carrier.

• **Heat Treatment and DNA Preparation.** The cell suspensions are heated to reduce viability and inactivate nucleases. Once the temperature is reduced, two lytic enzymes are added and the sample carrier is loaded into the instrument with the required consumables. Once the operator enters strain-tracking and optional related information into a conveniently designed batch program, the instrument will automatically process the strains. Bacterial cells are lysed with a series of lytic enzymes and the released DNA is cut or digested with a restriction endonuclease, EcoRI.

• **Separation and Transfer.** The DNA restriction fragments are size-separated by electrophoresis on an agarose gel. Each gel cassette contains 13 wells. The DNA samples under investigation are placed in eight of the wells; refer-

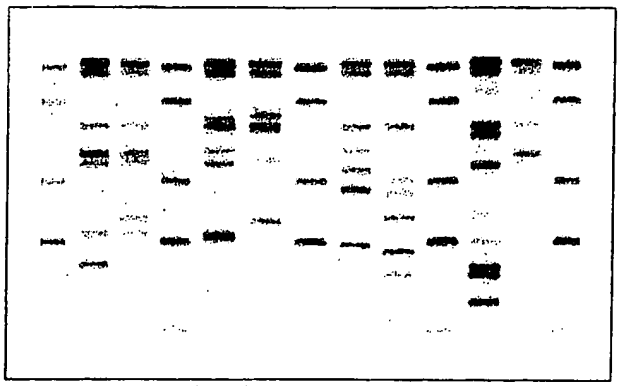

Fig.3—**Typical Batch Image**

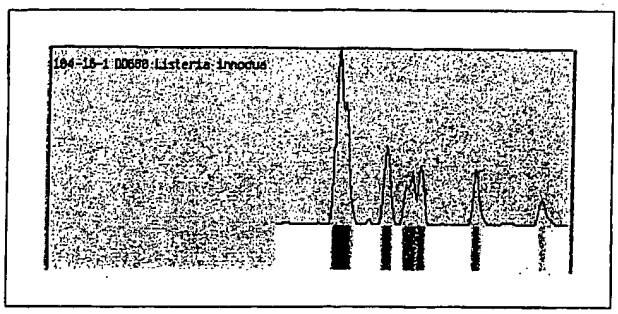

Fig.4—**Typical Pattern**

ence DNA of known molecular weights occupies the remaining five wells. As the DNA is electrophoretically separated and resolved in the gel, a nylon membrane moves vertically against the gel, allowing fragments to be captured and immobilized on the membrane.

• **Membrane Processing.** After denaturation of the DNA on the membrane, it is hybridized with a labeled rRNA operon probe. The membrane is washed then treated with blocking buffer and an antisulfonated DNA antibody/alkaline phosphatase conjugate. Unbound conjugate is removed through a series of washes and a chemiluminescent substrate applied.

• **Image Detection and Analysis.** The membrane is heated (Kobos et al., 1995) and positioned in front of a customized CCD camera, which detects the light intensity of the targeted DNA fragments. The camera converts the patterns from luminescing DNA fragments to digital information. This image data (Fig. 3) is stored in the system computer's hard drive memory.

Software extracts information from the image. The software recognizes data lanes on the image and distinguishes between reference marker and sample lanes. The position and intensity of well-characterized marker fragments, run simultaneously with the unknown sample, allows the system algorithms to normalize the resulting output data.

After the automated system processes a batch of samples, it generates a pattern for each sample and marker lane using proprietary algorithms. The pattern for each lane consists of a series of light and dark bands which can also be represented as waveforms (Fig. 4).

The system statistically compares the output pattern to patterns obtained previously. This is a two-part operation. For identification, statistical analysis allows the conclusion that the unknown sample can or cannot be matched with known standards stored in the identification database and a classical taxonomic name applied. Those samples with matches above a fixed similarity threshold are identified; those below the threshold are not. For classification, the sample patterns are grouped with all existing pattern types run in the system to form RiboGroups. A separate similarity threshold is used to determine grouping. If a sample cannot join an existing RiboGroup because it falls below the threshold, the system creates a new RiboGroup for that sample.

Identification and Characterization

The system software contains a database of more than 500 strains/pattern types of four genera of the greatest importance to the food industry: *Listeria*, *Staphylococcus*, *Escherichia*, and *Salmonella*. The system matches samples against this database to assign identifications at a genus, species, and strain level. For purposes of characterization, the automated system compares a current sample in each run with every sample that was ever run on the system. Characterization is independent of identification. All samples run on the system are characterized automatically.

Results of the statistical comparison tell the user immediately if an indistinguishable organism has been processed by the system previously. This information may suggest a route of contamination or provide insight into the efficacy of a sanitation process. The system then produces a report that characterizes and identifies each sample in the batch.

In addition, investigators can create individual databases of organisms of interest to a particular site. This feature permits the matching of bacteria from different sources and association of the patterns with a user-defined taxonomic identification. This is called a Custom Identification database.

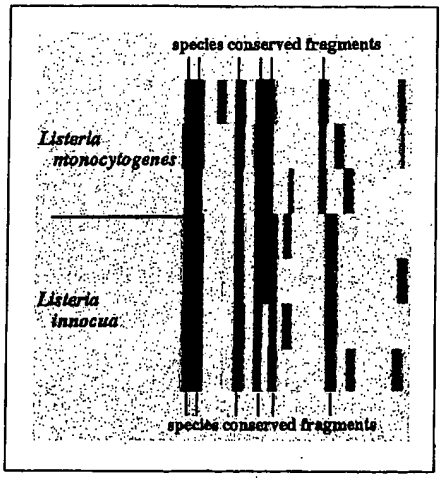

Fig.5—**Conserved Characters Distinguish Species of *Listeria*.** *The conserved characters that distinguish* L. monocytogenes *from* L. innocua *are indicated above. Variations in the fragments in the sixth position at the right show the strongest differentiation*

• **Distinguishing Species.** The results of more than 10,000 strains processed at DuPont labs have demonstrated that certain characters, ribosomal RNA fragments, are highly conserved at the genus and species level (Bruce et al., 1995; Hubner et al., 1995). For example, Fig. 5 compares size separation of these fragments within three strains of *Listeria monocytogenes* with four of *L. innocua*. While the two species share a certain amount of rRNA fragment information, clearly each species contains conserved bands (fragments) that are present at defined positions in one species while absent in other species, as illustrated in Fig. 5. The identification of each species for this comparison was verified by classical testing with cytochrome oxidase, catalase, and CAMP.

The degree of conservation shown in Fig. 5 suggests that sets of rRNA fragments, conserved as a group, are sufficient to identify the genus and

species of most bacteria. The remaining non-conserved polymorphic fragments constitute characters which differentiate strains within a particular species.

• **Distinguishing Strains.** Fig. 6 shows an example of seven RiboGroups. A RiboGroup is a set of closely related patterns that are mathematically indistinguishable from one another by the system. The RiboGroup patterns in Fig. 6 represent composite patterns for all members of the groups. For example, in Fig. 7, RiboGroup 780-S-5 is based on an average value for 10 constituent patterns. In Fig. 6, six of the eight EcoRI fragments (A, B, C, D, F and H) appear to be evolutionarily conserved across these strains of *L. monocytogenes*. The polymorphic fragments appear at several discrete positions, indicating strain variations within the species.

In our studies, we have found greater diversity in *Listeria* than is possible to observe by classical serology (Bruce, et al., 1995, Webster et al., 1994). Such high characterization resolution can simplify the job of identifying infectious sources in food production or other environments where contamination can be problematic.

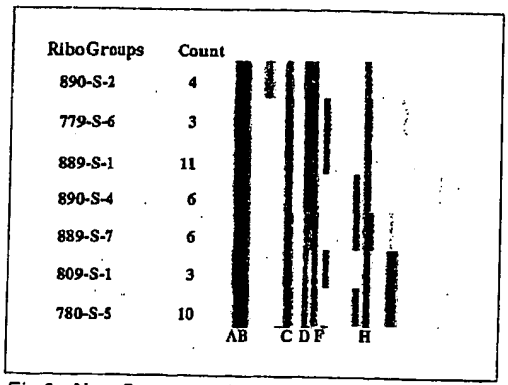

Fig.6—**Non-Conserved Characters Differentiate Strains of *L. monocytogenes*.** *The various polymorphic fragments represent diversity within a species and can be used to differentiate strains. The conserved fragments (A-H) define the species. the count number indicates the number of individual strain patterns that comprise each RiboGroup (see Fig. 7)*

Applications

The system has useful applications in a variety of settings. These include epidemiological uses as well as in food processing operations.

• **Epidemiology.** In 1994, four separate outbreaks of listeriosis involving cattle, sheep, and goats were examined to determine the relationship of the involved animals to their feed sources (Wiedmann et al., 1995). Strains of *Listeria monocytogenes* that were isolated from the animals diagnosed with encephalitic disease and from their feed were processed using the automated system.

Fig. 6 illustrates a subset of the RiboGroup patterns obtained for all isolates in the study. Within each outbreak, two to six populations of strains were found and each strain was identified as *L. monocytogenes*. In three out of four outbreaks, the same strain appeared in silage and isolates taken from infected animals, strongly implicating the silage feed as the source of infection. Within one outbreak, the system did not link the strains from the food source and the clinical sample, suggesting the cause of infection may have been from an alternative source. Classical methods were unable to differentiate between these *L. monocytogenes* strains, making absolute association between food source and clinical isolate impossible.

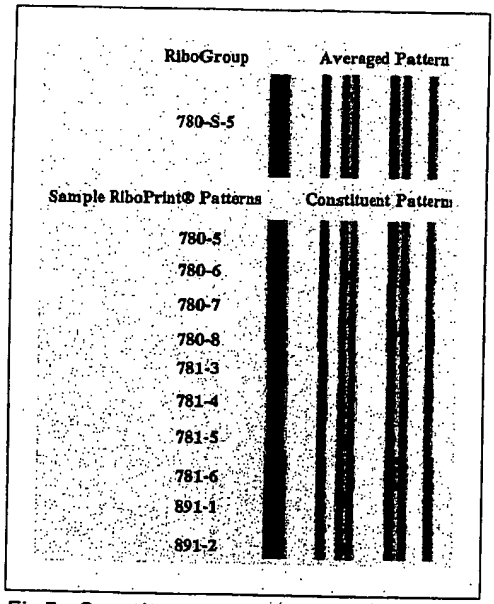

Fig.7—**Constituent Samples in a Typical RiboGroup.** *The ten sample patterns are mathematically averaged to describe the RiboGroup pattern. The RiboGroup pattern is named for the lowest-numbered strain pattern*

• **Food Processing.** A food processing plant that produces packaged, ready-to-eat foods discovered *Staphylococcus* contamination in two products as a result of routine microbial testing. Since *Staphylococcus* is often present in many parts of a given plant, the Quality Assurance/Control team was challenged to pinpoint the source of contamination in the finished products. Conventional microbial testing techniques did not yield conclusive results: the suspect organism, *Staphylococcus epidermidis*, showed up in many sources, and the plant staff was unable to determine the relationship between these sources and the isolates from the products.

The QA/QC team used the automatic ribotyping system to extract below-species level information for the various *Staphylococcus* isolates (Fig. 8). The patterns that were generated enabled them to determine that: (1) the *S. epidermidis* isolated from the contaminated food product was different from that found in the raw material but indistinguishable from one other pattern; and (2) only a single source in the plant yielded a pattern that matched those from the contaminated products. That source was the hands of one of the plant employees.

As a result, corrective measures proved simple and inexpensive. The team revised the rules governing hand-washing and glove usage, and took steps to minimize the transfer of bacteria between raw material handling and post-processing sections of the plant. Using the automated system to quickly and effectively pinpoint the contamination source, the plant was able to avoid the expense of a costly full-scale shutdown and decontamination effort. A company in Europe bought fermented and cooked meat products in bulk, then prepared pizza toppings and sauces for supply to the food service and retail sector. Suddenly, Health Department inspectors reported that they had isolated *Listeria monocytogenes* from the pizza toppings. The company's laboratory was not able to detect the organism immediately after production, but did concede that growth could occur during the extended chilled distribution and storage of the product. A detailed microbiological analysis of the factory showed that raw meats contained the organism of concern. Before removing suppliers from their list, the company decided to further analyze their findings using the automated system technology. Over a period of three days, the system processed the isolates and determined that, although some raw materials were indeed contaminated with *Listeria monocytogenes*, the patterns were different from those found by the Health Department. The

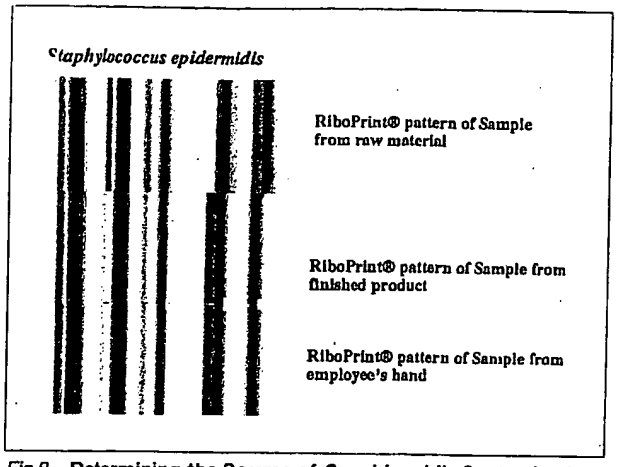

Fig.8—**Determining the Source of *S. epidermidis* Contamination.** *Samples from raw materials and the contaminated finished product were processed in the system. The patterns did not match. Patterns from samples taken in the environment did match. The source of contamination was an employee*

investigators shifted emphasis to analysis of larger samples of finished product and found very low numbers of *Listeria monocytogenes* but this time, the patterns matched those found by the health inspectors (Fig. 9).

Further investigation showed that the only area of the factory contaminated with this matching type of *Listeria monocytogenes* was the cleaning/hygiene room, where all the chemicals and detergents were stored. Cleaning was contracted out to a separate company who worked at night, so the team decided to monitor the cleaning practices during the following week. They found that the poorly trained hygiene crew cleaned and sanitized the raw meat storage area, then moved to the pizza preparation area without implementing any decontamination steps. This lack of hygiene precautions resulted in them carrying the *Listeria monocytogenes* contamination into the sensitive area of the factory. The manufacturer changed cleaning contractors and solved the problem.

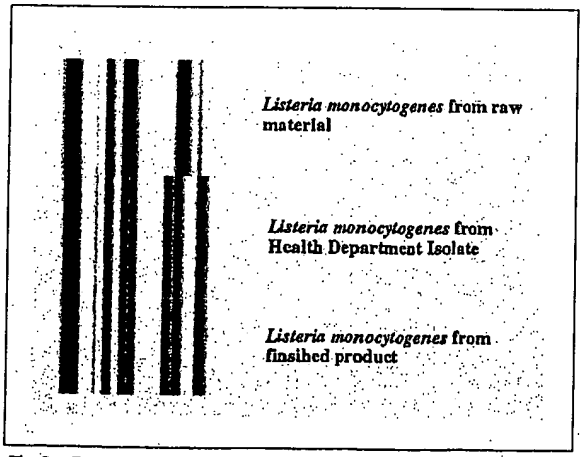

Fig.9—**Determining the Source of *L. monocytogenes* Contamination in Pizza Topping.** *Samples from raw materials and the health department isolates showed* L. monocytogenes. *But the patterns did not match. Further sampling of the environment revealed samples from the cleaning/hygiene rooms that did match those isolated by the health department*

Valuable Tool

The automation of bacterial characterization has begun to yield substantial value in many areas of microbiological investigation. This article has concentrated on two primary benefits of the equipment. The first is speed in obtaining accurate, reliable results. Characterization and identification of a bacterium to the strain level is achieved in about 8 hr. The second important benefit is the high information content of the result thus obtained and archived. The molecular basis of the analysis has enabled research scientists to reproducibly characterize more than 1000 strains of a single organism, *Listeria monocytogenes*. The information produced is not subject to the variability inherent in much conventional testing, and affords a reliable degree of characterization or identification.

These benefits make the automated system an indispensable tool in a variety of applications. In food processing, it is now possible, in the space of a few hours, to precisely pinpoint the source of a microbial contamination, be it a pathogen or a spoilage organism. This focuses investigation and remediation, avoiding time-consuming investigations and costly plant shutdowns. In dairy farming and other industries involving the management of animals, diseases potentially affecting large herds can be traced quickly to their source, simplifying the job of eliminating infections.

Another application, not touched on in detail here, is the monitoring of beneficial microorganisms. For example, in various kinds of dairy production, known and often proprietary cultures of organisms are added to yield a distinctive end product, such as cheese or yogurt. It is of obvious interest to want to monitor starter cultures to assure the production of consistent, high-quality product.

Finally, most of the applications described here have relied on a database of organisms provided with the automated system to assign a classical taxonomic identification. However, as previously mentioned, it is possible with this system to build custom databases from culture collections, using microorganisms of special interest to the user. The automation of the process and the features described above have allowed ribotyping to expand into every area of microbiology.

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Microbiology

Sets of *Eco*RI fragments containing ribosomal RNA sequences are conserved among different strains of *Listeria monocytogenes*

(bacteria/ribotyping/classification/identification)

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ABSTRACT **To classify *Listeria monocytogenes* using taxonomic characters derived from the rRNA operons and their flanking sequences, we studied a sample of 1346 strains within the taxon. DNA from each strain was digested with a restriction endonuclease, *Eco*RI. The fragments were separated by gel electrophoresis, immobilized on a membrane, and hybridized with a labeled rRNA operon from *Escherichia coli*. The pattern of bands, positions, and intensities of hybridized fragments were electronically captured. Software was used to normalize the band positions relative to standards, scale the signal intensity, and reduce the background so that each strain was reproducibly represented in a data base as a pattern. With these methods, *L. monocytogenes* was resolved into 50 pattern types differing in the length of at least one polymorphic fragment. Pattern types representing multiple strains were recorded as the mathematical average of the strain patterns. Pattern types were arranged by size polymorphisms of assigned rRNA regions into subsets, which revealed the branching genetic structure of the species. Subtracting the polymorphic variants of a specific assigned region from the pattern types and averaging the types within each subset resulted in reduced sets of conserved fragments that could be used to recognize strains of the species. Pattern types and reduced sets of conserved fragments were conserved among different strains of *L. monocytogenes* but were not observed in total among strains of other species.**

Strains of *Listeria monocytogenes* are classified into the taxon based on genotypic and phenotypic similarities (1, 2). A general method for classification and identification of strains by using DNA restriction fragments containing portions of rRNA operons has been described (3, 4). This method has been applied to the genus *Listeria* (5) and to *L. monocytogenes* (6), demonstrating its utility for classifying, identifying, and typing strains.

We have described a standard method for species description by using conserved sets of species-specific rRNA gene restriction endonuclease-derived fragments (7). In the present study, >1000 strains of *L. monocytogenes* were characterized by using *Eco*RI fragments containing sequences complementary to an rRNA operon from *Escherichia coli*. The pattern structure of the species was described in detail by the use of fixed electrophoretic conditions, fragment standards, electronic imaging, and software for mobility normalization. In addition, we introduced the use of continuous-scale relative intensity in recording patterns from ~9000 strains of ~200 species. The *L. monocytogenes* patterns were arranged into the taxonomic structure by the use of squared correlation values (8) and visual assessment. We assigned letter names to the rRNA regions, each containing a given part of a given operon, and differentiated strains by restriction fragment length polymorphisms of those regions. The structure of patterns revealed similarities, reduced sets of conserved fragments shared by multiple pattern types within subsets of the sample set, and the progression of strain variation through the classically described species.

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MATERIALS AND METHODS

Classical Characterizations. The following attributes were determined as described (9): cytochrome oxidase activity, catalase activity, and hemolysin activity enhancement (CAMP test). Biochemical profiles were obtained with the Micro-ID Listeria System (Organon Teknika–Cappel). The strains, representing all serotypes, were isolated from a wide variety of animals, foods, and environmental niches.

Reagent Preparation. The *rrnB* rRNA operon from *E. coli* (10), inserted and replicated in pGEM, was digested with *Eco*RI before labeling. After digestion, the DNA was precipitated, dissolved in water to a concentration of 0.8–1.0 mg/ml, denatured by immersion in a boiling water bath, and chilled on ice. To label the DNA by sulfonation (11), a volume of 2.0 M sodium bisulfite solution (pH 5.6) equivalent to one-half of the DNA-solution volume and a volume of 1.0 M methoxylamine hydrochloride solution (pH 6.0) equivalent to one-eighth of the DNA-solution volume were added. The samples were mixed, and the pH of the solution was adjusted to 6.0 or less with HCl before incubating overnight at 30°C. Labeling reagents were removed by Sephadex G-25 (Pharmacia) column chromatography.

A conjugate of anti-sulfonated DNA monoclonal antibody (Orgenics, Yavne, Israel) and alkaline phosphatase (AP) (Boehringer Mannheim) (12, 13) was prepared by adding 15 times molar excess of *N*-succinimidyl-4-(*N*-maleimidemethyl)-cyclohexane-1-carboxylate (Pierce) at 10 mg/ml in dimethyl sulfoxide to 50 mg of dialyzed antibody in 10 mM sodium phosphate/300 mM NaCl, pH 7.0. The mixture was incubated in the dark in a 25°C water bath for 30 min and then placed on ice to stop the reaction. Unreacted cross-linking reagent was removed by Sephadex G-50 column chromatography (Pharmacia). Fractions containing activated monoclonal antibody were pooled, and the molar concentration was determined.

N-Succinimidyl-*S*-acetylthioacetate (Pierce) at 10 mg/ml in dimethyl sulfoxide was added to an AP solution (Boehringer Mannheim) at a 15 times molar excess. The amount of AP (10 mg/ml) used was that required to produce a 1:1.6 (wt/wt) ratio of monoclonal antibody to AP. The mixture was incubated in the dark in a 25°C water bath for 30 min, and the reaction was stopped by placing the mixture on ice. The sulfhydryl groups were deprotected by adding 500 μl of 1.0 M hydroxylamine for every 10 mg of AP and placing the solution in the dark in a 25°C water bath for 30 min. This reaction was also stopped by placing the mixture on ice and was followed by the removal of the unreacted cross-linking agent by Sephadex G-50 column

Abbreviation: AP, alkaline phosphatase.
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chromatography. Fractions containing activated AP were pooled, and the molar concentration was determined.

AP containing sulfhydryl groups was conjugated to the maleimide-activated monoclonal antibody by mixing the two solutions at a ratio of 1 mg of monoclonal antibody:1.3 mg of AP and incubating the mixture in the dark in a 25°C water bath for 2 hr. This reaction was stopped by adding 18 μl of 0.1 M *N*-ethylmaleimide for every 10 mg of monoclonal antibody and incubating the mixture in a 25°C water bath for 30 min. The conjugate was concentrated by using an Amicon ultrafiltration stirred cell, model 8050, with a YM100 Diaflo membrane and purified by Sephacryl S300 column chromatography (Pharmacia) using 50 mM Tris/100 mM NaCl, pH 8.0. The purified conjugate was stored at −20°C after 1:1 (vol/vol) dilution with storage buffer (50 mM Tris·HCl, pH 8.0/150 mM NaCl/1% bovine serum albumin/2 mM $MgCl_2$/0.2 mM $ZnCl_2$).

Lysis of Bacteria and DNA Extraction. Strains grown overnight in 3 ml of brain heart infusion broth (Difco) were collected by centrifugation in a 1.5-ml tube, resuspended in 200 μl of 10 mM Tris·HCl, pH 8.0/10 mM NaCl/50 mM EDTA, pH 8.0 and heated at 75°C for 10 min. Cells were treated with 30 μl of *N*-acetylmuramidase at 1 mg/ml (Seikagaku America, Rockville, MD), 30 μl of lysozyme at 20 mg/ml (Sigma), 5 μl of lysostaphin at 5000 units/ml (Sigma), and 5 μl of RNase, DNase free at 2000 units/ml (Boehringer Mannheim) at 37°C for 15 min, followed by addition of 40 μl of crude achromopeptidase at 20 mg/ml (Wako Pure Chemical, Osaka) and 15 min of additional incubation at 37°C. After the addition of 100 μl of 10% SDS (Bio-Rad) and 126 μl of proteinase K at 10 mg/ml (Boehringer Mannheim), the solution was incubated at 65°C for 30 min. The cell lysate was transferred to a 1.5-ml phase-lock gel I light centrifuge tube (5 Prime → 3 Prime, Inc.), extracted with phenol/chloroform (Applied Biosystems), and the DNA was ethanol-precipitated with 3 M NaOAc. The precipitated DNA was collected by centrifugation, and the ethanol was removed. The pellet was washed with ethanol and air-dried for at least 15 min. The DNA was resuspended in 500 μl of 1× TE buffer (10 mM Tris·HCl, pH 8.0/1 mM EDTA, pH 8.0) and 5 μl of DNase-free RNase (Boehringer Mannheim), incubated at 37°C for 4 hr and stored at 4°C.

DNA Digestion and Electrophoresis. After determination of nucleic acid concentration by absorbance at 260 nm, 5 μg of DNA was diluted to 158 μl with water, 40 μl of 5× *Eco*RI buffer [1× *Eco*RI buffer is 100 mM Tris·HCl (pH 7.5), 50 mM NaCl, 10 mM $MgCl_2$, 10 μg of bovine serum albumin], and 2 μl of *Eco*RI at 50 units/μl (Boehringer Mannheim) were added, and the DNA was digested overnight at 37°C. Loading solution was prepared by mixing 2.75 ml of a 0.25% bromophenol blue/0.25% xylene cyanol/25% Ficoll solution with 0.55 ml of 0.5 M EDTA (pH 8.0), and 2.5 μl was added to 17.5 μl of digested DNA. Four microliters of the resulting solution (87.5 ng) was applied per lane to a 0.8% agarose (SeaKem GTG, FMC) gel prepared with TTNE buffer (20 mM Tricine/50 mM Tris base/5 mM NaOAc/10 mM EDTA, pH 8.65) and electrophoresed at 40 V for 2.75 hr (Horizon 58 mini-gel, BRL). To ensure accurate data extraction, size standards consisting of pooled *Bgl* I, *Cla* I, and *Ssp* I digests of pKK3535 (10) were electrophoresed in lanes adjacent to samples yielding eight samples and five standards per gel.

Electrophoretic Transfer, Denaturation, and UV Cross-Linking. After electrophoresis, the DNA was transferred to a membrane (N04HYT, Micron Separations, Westboro, MA) with a Hoefer transfer unit (TE 22) via a 1-hr electrophoresis at 1.0 A in TTNE buffer at 4°C. After transfer, the membrane was rinsed in TTNE buffer to remove residual agarose and placed, DNA-side up, on a sheet of 3MM paper (Whatman) saturated with the denaturing solution (0.2 M NaOH/1.5 M NaCl). After 2.5 min, the membrane was transferred to a second blotter paper saturated with TTNE buffer for 15–20 s. The membrane was dried for 30 min under a heat lamp, DNA-side up, on a piece of 3MM paper. The DNA was cross-linked to the membrane with a Bios crosslinker 312T (Bios) at an automatically timed dose of 0.6 J/cm².

Hybridization and Detection. Each membrane was prehybridized with 5 ml of hybridization solution (denatured, sonicated, salmon-sperm DNA at 125 μg/ml/0.5 M NaCl/1% SDS) for 10 min at 66°C in a roller bottle apparatus. This solution was then replaced with 6 ml of probe solution (hybridization solution containing 1.5 μg of heat-denatured sulfonated DNA). After overnight hybridization at 66°C, the probe solution was decanted, and 20 ml of 66°C wash buffer (0.5 M NaCl/1% SDS) was added. The bottle was returned to the hybridization oven for 15 min. This treatment with wash buffer was repeated for a total of four washes. The membrane was removed from the bottle, placed on a blotter, DNA-side up, and dried for 10 min at 30°C.

Before application of the conjugate, each hybridized membrane was submerged in 20 ml of freshly prepared blocking buffer [30 g of skim milk powder (Difco) per 100 ml of 25 mM NaCl/50 mM Tris·HCl, pH 7.5/1 mM EDTA/0.3% Tween 20] and placed on a rocker in a 30°C incubator. After 30 min, the buffer was decanted and replaced with an amount of conjugate (based on titer, typically 1:100 to 1:300) in blocking buffer. The tray was returned to the rocker for an additional hour. This incubation was followed by three successive 5-min washes (20 ml of 0.5 M NaCl/0.3% Tween 20) at room temperature. The membrane was then washed three times for 5 min each with assay buffer (50 mM sodium bicarbonate/carbonate and 1 mM $MgCl_2$, pH 9.5). After decanting the final assay-buffer wash, 20 ml of assay buffer containing 220 μl of phosphate phenyl dioxetane (10 mg/ml) (Lumigen, Southfield, MI) was added for each membrane. The tray was covered and held at room

Table 1. Invariant fragment sizes for the base pattern type and for each subset of *L. monocytogenes*

Name	Strains, no.	Strains, %	Sizes of fragments containing rRNA operons, kbp							
			H	G	F	E	D	C	B	A
dd 0566 (base type)			9.0	6.2	5.5	5.2	5.0	4.0	2.3	2.1
E 5.2	438	32.5	9.0	v	5.5	5.2	5.0	4.0	2.3	2.1
E 5.3	10	0.7	9.0	v	5.5	5.3	5.0	4.0	2.3	2.1
E 9.2	499	37.1	9.0	v	5.5	9.2	5.0	4.0	2.3	2.1
E 11.2	227	16.7	9.0	v	5.5	11.2	5.0	4.0	2.3	2.1
E 11.2-C	2	0.1	9.0	v	5.5	11.2	5.0		2.3	2.1
E 5.2-D	14	1.0	9.0	v	5.5	5.2		4.0	2.3	2.1
G 6.2, H 9.0	127	9.4	9.0	6.2	v	5.2	5.0	4.0	2.3	2.1
G 8.1, H 7.1	11	0.8	7.1	8.1	v	5.2	5.0	4.0	2.3	2.1
G 5.8, H 7.1	3	0.2	7.1	5.8	v	5.2	5.0	4.0	2.3	2.1
E/G 5.8, H 7.1	2	0.1	7.1	5.8	v	5.8	5.0	4.0	2.3	2.1
Thermal control	13	1.0	9.0	6.2/3.2	5.5	5.2	5.0	4.0	2.3	2.1

v, Variable.

temperature for 5 min with rocking, followed by 10 min without rocking. The membrane was then taped DNA-side up on blotter paper and dried in a 30°C incubator for a minimum of 30 min. The membrane was then removed from the blotter paper and heated in a 700-W microwave oven on the high setting for 10 s. The chemiluminescent image was recorded electronically and analyzed as described (7). The initial results, electronically recorded images of membranes showing *Eco*RI fragments and standards separated by size and labeled by hybridization with the probe containing the rRNA operon, were processed to extract and normalize the pattern of fragment bands for each lane of data, and the patterns were entered into a data base.

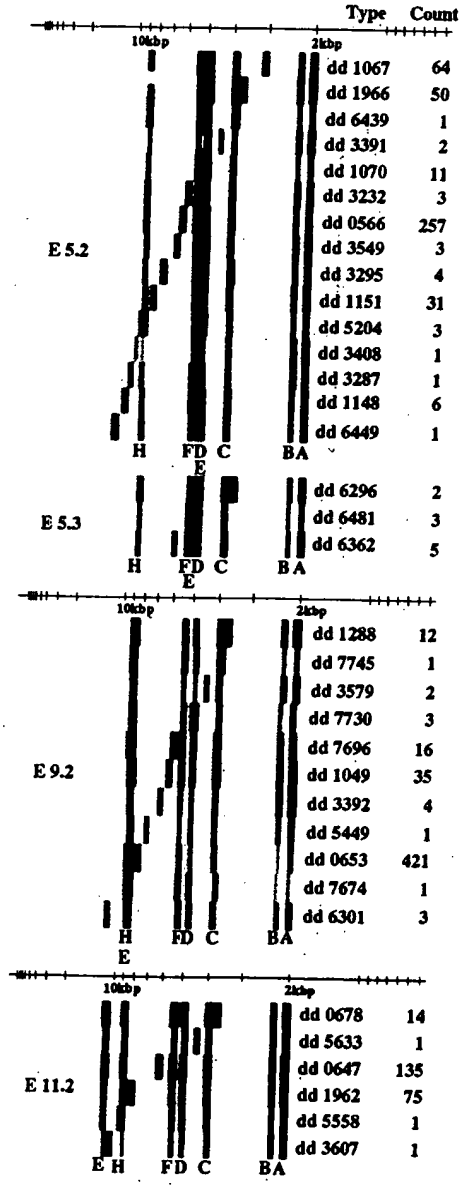

FIG. 1. *L. monocytogenes* types (*Eco*RI) arranged in subsets according to the indicated sizes (kbp) of E variants. The types within each subset were sorted with the G-fragment sizes increasing from small to large. The type name and the number of strains in each type are shown. DD 0566, ATCC 15313; nomenclatural type; DD 0647, ATCC 19118; and DD 0653, ATCC 19115.

RESULTS

Pattern Types. The patterns were arranged by similarity through the use of squared correlation values and visual assessment to determine nearest neighbors. These patterns, representing 1346 strains, revealed sets of hybridized fragments that were conserved among different strains of *L. monocytogenes* but were not observed as a set in any other of the >200 species tested. Patterns that were indistinguishable within the experimental error were averaged, and the average was stored as a pattern type. Any specific pattern representing a single strain also constituted a pattern type. With this approach the diversity within *L. monocytogenes* was resolved into 50 distinct types. Thirty-four types represented two or more strains. Each type that represented a single strain differed from its nearest neighbor by the position of a single band. Each type (prefix dd) was assigned the number of the strain first represented by the pattern type (prefix DD).

Formation of Coherent Subsets. Each hybridized fragment of a given size in the *L. monocytogenes* patterns was considered to be a taxonomic character, and the frequencies of occurrence of these fragments in the patterns of *L. monocytogenes* strains were determined. Type dd 0566 incorporated the hybridized fragments with the highest frequencies of occurrence and was designated as the base type for comparison of suggested polymorphic fragments. Table 1 includes the size of each of the fragments in dd 0566. These fragment bands were lettered sequentially on the basis of their relative positions in the base type.

Once the base type was established, other types were described by the size polymorphisms of the detected fragments. Accordingly, the types were grouped by squared correlation values and visual arrangement of the suggested size polymorphisms of a given fragment into coherent subsets. Within these subsets, the coherence is that all bands are fixed in position except one.

Fig. 1 shows four subsets. In each of these subsets, the E fragment was fixed at a different size, and G fragments were polymorphic. The bands with fixed positions in the subset are labeled; the G fragments are apparent by their different sizes. These four subsets represent the dominant structure of the species. The first subset, labeled E 5.2, included the base type (dd 0566) and other patterns that differed from the base pattern only in the restriction fragment length polymorphisms of the G region. The E 5.3, E 9.2, and E 11.2 subsets also represented E-variant subsets with some of the same multiplicity of G-fragment sizes. The two types in subset E 11.2-C (Fig. 2) differed from dd 0647 and dd 1962 by a 4.0-kbp C-fragment band deletion. Subset E 5.3-D included dd 6323, which differed from dd 6296 (Fig. 1, E 5.3 subset) by a 5.0-kbp D-fragment band deletion.

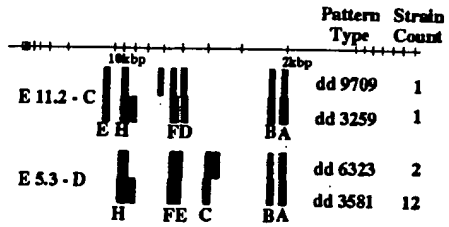

FIG. 2. Additional *L. monocytogenes* types (*Eco*RI) arranged in subsets according to the indicated sizes (kbp) of E variants and the noted deletions or unseen polymorphisms. The types within each subset were sorted with the G-fragment sizes increasing from small to large. The type name and the number of strains in each type are shown.

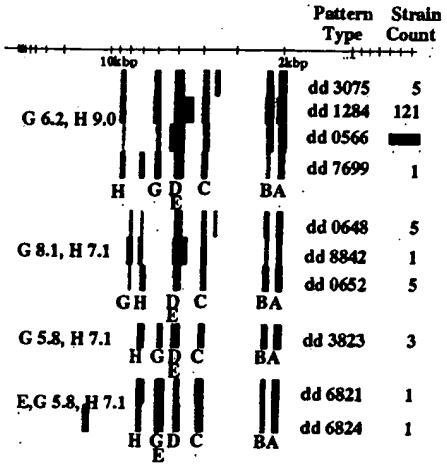

FIG. 3. Additional *L. monocytogenes* types (*Eco*RI) arranged in subsets according to the indicated sizes (kbp) of G, H, and E variants. The last three pattern types are considered as a single subset. The types within each subset were sorted with the F-fragment sizes increasing from small to large. The type name and the number of strains in each type are shown. DD 0566, ATCC 15313; DD 0648, ATCC 19114; and DD 0652, ATCC 19116.

Fig. 3 shows additional subsets. In the first two of these subsets, G and H fragments were of different fixed sizes. The two G-fragment sizes were the most frequently occurring, as seen in the strain counts in Fig. 1 (dd 0566, dd 6362, dd 1049, and dd 0647 for G 6.2; dd 1151, dd 0653, and dd 1962 for G 8.1). The bands with fixed positions in the subset are labeled, and the F fragments were apparent by their different sizes. Type dd 0566 fits into both the E 5.2 subset of Fig. 1 and the G 6.2, H 9.0 subset of Fig. 3 and links the subsets. The G 8.1, H 7.1 subset differed from the previous subset in the position of G and H. Because the H 7.1 polymorphism had not been observed in any other subsets, all strains in this subset were classically retested and verified as *L. monocytogenes* (cytochrome oxidase, negative; catalase, positive; CAMP, positive with *Staphylococcus aureus* ATCC 25923, and Micro-ID octal code, 44044).

The patterns labeled G 5.8, H 7.1 and E/G 5.8, H 7.1 (Fig. 3) differed from the G 6.2, H 9.0 subset in the variant positions of fragments G and H and of fragments E, G, and H, respectively. The G 5.8, H 7.1 and E/G 5.8, H 7.1 subsets had two (G and H) of six bands with a low frequency of occurrence for *L. monocytogenes*. The 5.8-kbp fragment (G) had a high frequency of occurrence in *Listeria innocua* (Fig. 4). However, these subsets shared the H 7.1 fragment with the G 8.1, H 7.1 subset, and this fragment is not present in the adjacent *L. innocua* patterns. Correlation analysis (data not shown) indicated that the represented strains were closer to *L. monocytogenes* than to *L. innocua*. To verify the identification, all strains in this subset were also retested classically. These strains were as follows: cytochrome oxidase, negative; catalase, positive; and CAMP, positive, as expected for *L. monocytogenes*. However, the rhamnose reaction in the Micro-ID system was negative and produced an octal code of 44040. This result is atypical, although rhamnose-negative strains of *L. monocytogenes* have been reported (14).

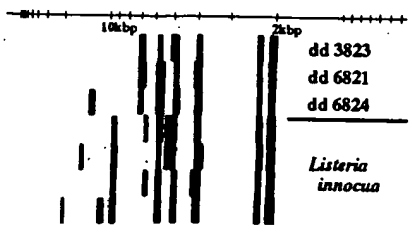

FIG. 4. Three distal pattern types of the structure of *L. monocytogenes*, shown with some of the adjacent edge of *L. innocua* pattern types.

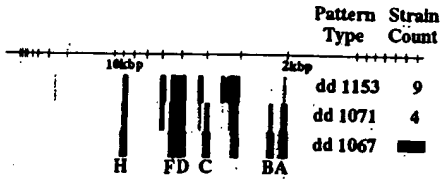

FIG. 5. Types affected by growth temperature. When the strains in type dd 1153 were grown either at 20°C or at 30°C, pure cultures presented at least two populations, as shown by the polymorphic duplication of some bands. Similar results were obtained with strains of type dd 1071, but the relative intensities of the bands differ, especially C, B, and A. When strains from either type were grown at 42°C, they produced patterns matching dd 1067. The type name and the number of strains in each type are shown.

The first two types in Fig. 5, dd 1153 and dd 1071, constitute the last subset; the patterns from strains of these types were affected by the growth temperature. These pattern types were similar to dd 1067 (included in Fig. 5 for comparison), except that at least two different genetic population types were present in the pure cultures, as shown by the same regions manifested in multiple bands. The different population types occurred when the cultures were grown either at 20°C or at 30°C. When the cultures were grown at 42°C, the resulting patterns matched the dd 1067 pattern type.

Table 1 summarizes the fragment sizes for each of the subsets.

Reduced Sets of Conserved Fragments. A reduced set of conserved fragments was constructed for each of the coherent subsets by removing the information of the highly polymorphic region from the pattern types and averaging the fragment bands occurring at 100% frequency in each subset. The G 5.8, H 7.1 and E/G 5.8, H 7.1 subsets were combined into one for this purpose. Fig. 6 shows the nine reduced sets of conserved fragments along with their descriptive names and strain counts.

DISCUSSION

Fragment Conservation. The survey of *L. monocytogenes* described here revealed similarities shared by strains of the

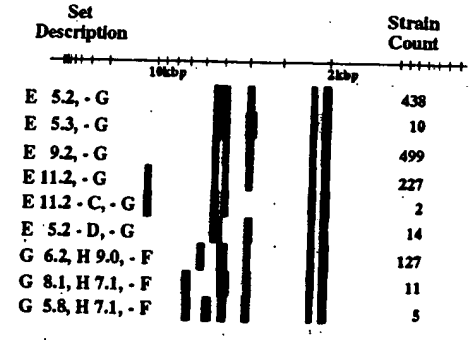

FIG. 6. Reduced sets of conserved fragments. By using the positions of the six or more bands that occurred at 100% frequency in each of the coherent subsets, nine reduced patterns were created. These reduced patterns were conserved among the strains of *L. monocytogenes* but were not observed as a set among the strains of the other species tested.

species and greater diversity than seen in classical serotyping (9). This description was made possible by use of a charge coupled-device camera detection of a light-emissive label (7), strict normalization of band positions relative to standards, and use of the full-scale normalized signal intensity in the computerized analysis. The conservation and strain variation permitted the data from 1346 strains, 50 pattern types, to be further reduced to nine minimal sets of fragments for identification of *L. monocytogenes*. Each minimal set was viewed as an ensemble of positive taxonomic characters with a related frequency of occurrence in the sample population (Fig. 6). The minimal sets, further sharing some of the fragment sizes, created a reduced definition of the species.

The cohesiveness of the total set of the 50 pattern types was verified by the observation that each pattern type differed from some other pattern types in the position of a single polymorphic fragment band. An exception was the step into the H-variant subsets, where a double polymorphic change was observed partitioning the strains branching toward *L. innocua*. A relationship of these strains was seemingly implied by the arrangement of pattern types, especially in the G-region polymorphisms shown in each subset of Fig. 1. However, the pattern types in these subsets could be rearranged into a number of different subsets, each explicitly showing the polymorphisms of the E region and G region conserved within each subset.

Fig. 5 includes two patterns types of strains that originally appeared to differ from the rest of the species. That the patterns in this subset could be recognized by using the reduced set of conserved fragments from subset E 5.2 and that patterns matching dd 1067 resulted when cultures were grown at higher temperatures suggested that this subset is intrinsically related to the E 5.2 subset.

Coherent Subsets. The similarities of the other 48 pattern types were made more apparent by the formation of coherent subsets. The E 5.2, E 5.3, E 9.2, and E 11.2 subsets (Fig. 1) differed from the base pattern, dd 0566, in the positions of both E variants and G-fragment sizes. Subsets E 5.3-D and E 11.2-C (Fig. 2) were viewed as low-frequency branches from the related E-variant subsets. Classical bacteriology recognizes that conserved attributes may not be present in all strains (15), and some minimal characters for identification may occur at a frequency of <100%. The base pattern was also a branch point for the F-variable subset (Fig. 3). The structure in Fig. 1 and the branches shown in Fig. 2 (all subsets) and 3 (subset G 6.2, H 9.0) include 1323 strains (98.3%).

The other subsets shown in Fig. 3 display greater divergence from the G 6.2, H 9.0 subset, with two and finally three regions with variant fragments. The decreasing number of variant fragments in common with the branching F-variable subset (G 6.2, H 9.0) seems to suggest divergence from the dominant structure of the species. However, the fragments do not appear randomly throughout the molecular size range but appear at discrete positions characteristic of the species. Correlation analysis indicated the G 8.1, H 7.1 subset was near the edge of the *L. monocytogenes* cluster. Classical methods confirmed the identification as *L. monocytogenes*. By DNA homology measurement, strain DD 0648 (ATCC 19114) is 72% related to the type strain of *L. monocytogenes* and 54% related to the type strain of *L. innocua* (1). In addition, several strains in type dd 0648 were associated with listeriosis in ruminants, a characteristic associated with *L. monocytogenes* rather than *L. innocua* (16).

The G 5.8, H 7.1 and E/G 5.8, H 7.1 types (Fig. 3) were considered together. Correlation analysis indicated that these subsets were clustered out from the branch point and in the direction of the *L. innocua* cluster. The negative rhamnose reactions and the octal codes from the Micro-ID kit were consistent with *L. innocua* (17). However, the positive CAMP results were consistent with *L. monocytogenes*. These separated branch subsets represented only a small percentage of the sample, and this last subset represented only five strains, apparently an edge of the species.

Reduced Sets of Conserved Fragments. Both the patterns and their embedded reduced sets of conserved fragments defined the species. On the basis of visual recognition of the E-variant band fragments, the first four patterns (representing reduced sets) in Fig. 6 could be further reduced to a single set by removing the information of the E polymorphic region (E 9.2, -G). The -C and -D patterns were viewed as branching strain variations with low frequencies of occurrence. This conservation progressed in stepwise fashion and branched at the F-variable patterns (Fig. 3) toward *L. innocua* (Fig. 4). The G 5.8, H 7.1 and E/G 5.8, H 7.1 conserved set was intermediate and was included provisionally within *L. monocytogenes* on the basis of the linkage to the previous set, the absence of an expected *L. innocua* fragment band and the positive CAMP reaction. The reduced sets of conserved fragments were recognized explicitly in the arrangement of patterns; this conservation was also recognized implicitly by similarity values.

We thank Dipti R. Shoop and Mong-Ching W. Ganfield for the labeled probe, conjugate, and discussion of methods.

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Gas Chromatography

HEWLETT PACKARD

Application Note 228-37

Gas-Liquid Chromatography of Cellular Fatty Acids as a Bacterial Identification Aid

Lindy Miller – Avondale Division

ABSTRACT

Bacterial fatty acids and culturing requirements are discussed. Sample preparation techniques for gas-liquid chromatographic analysis of fatty acids as methyl esters are outlined in detail. A high performance chromatographic system with operating conditions for use with a fused silica capillary column is described.

INTRODUCTION

Gas-Liquid Chromatography (GLC) is a valuable aid in the identification of bacteria. GLC analyses of the fatty acids present in the lipids of cells' membranous structures result in specific, reproducible fingerprints or profiles. By comparing whole cell fatty acid profiles, many closely related organisms may be differentiated (2,4,10). GLC is particularly useful for rapid identification of slow growing or non-fermentive bacteria (1,9,17,18). The analysis of cellular fatty acid profiles is also applicable to the identification of yeasts and other microorganisms (6,14). Distinctive fatty acid methyl ester (FAME) profiles have been determined for many organisms. Strains of a species show only minor quantitative variations in their FAME profiles. Closely related species or genera are distinguishable by qualitative differences (the presence or absence of fatty acids) or large quantitative differences in acid abundances. Reference profiles are generated by repetitive analyses of many strains within a species. Identification may then be made by matching FAME profiles of unknown organisms with reference profiles.

Fatty acid extracts can be prepared by saponifying whole bacterial cells in a strong base. After saponification, the cellular solution is acidified and the liberated fatty acids are then methylated to increase their volatility upon subsequent GLC analysis. The methyl esters of the fatty acids are extracted from the acidified aqueous solution. Before injection, the organic extract is washed with a dilute base. The washed extract can be analyzed by GLC with a nonpolar capillary column and a flame ionization detector.

The procedure is fairly simple and rapid. Batched samples can be prepared in approximately one hour. The actual total technologist's time is less than 4 minutes per sample. The GLC analysis of a sample can be done in less than 20 minutes. If an automatic sampler is used, the extracts can be analyzed without further technologist time. A desk top calculator can be programmed to identify the peaks by name and perform a library search for bacterial identification.

FATTY ACIDS

Most whole cell bacterial extracts contain fatty acids with carbon chain lengths of 10-20 carbons. These acids may vary in degree of unsaturation or branching and may also contain hydroxyl groups. (See Figure 1.) The

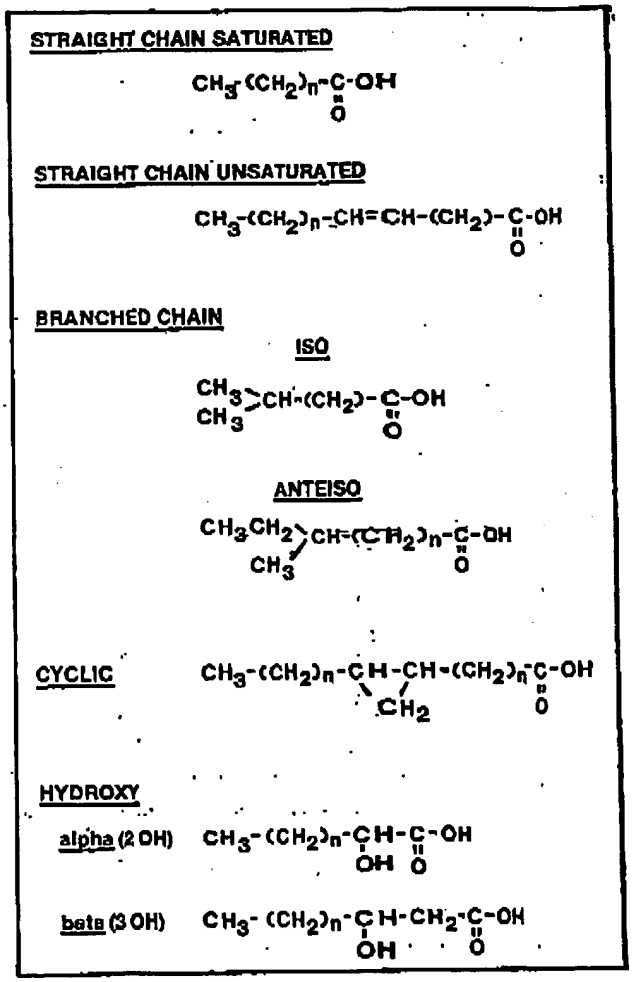


Figure 1. Bacterial Fatty Acids

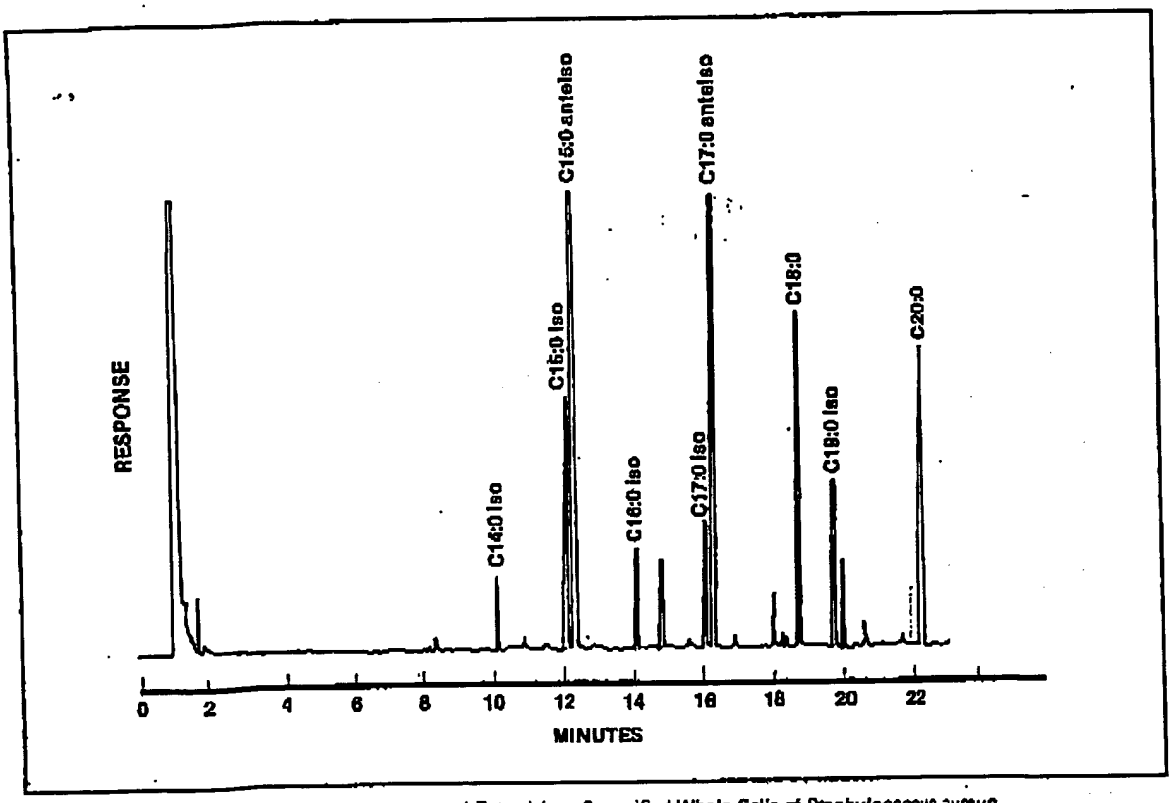


Figure 2. Gas Chromatogram of Fatty Acids (Methyl Esters) from Saponified Whole Cells of *Staphylococcus aureus*

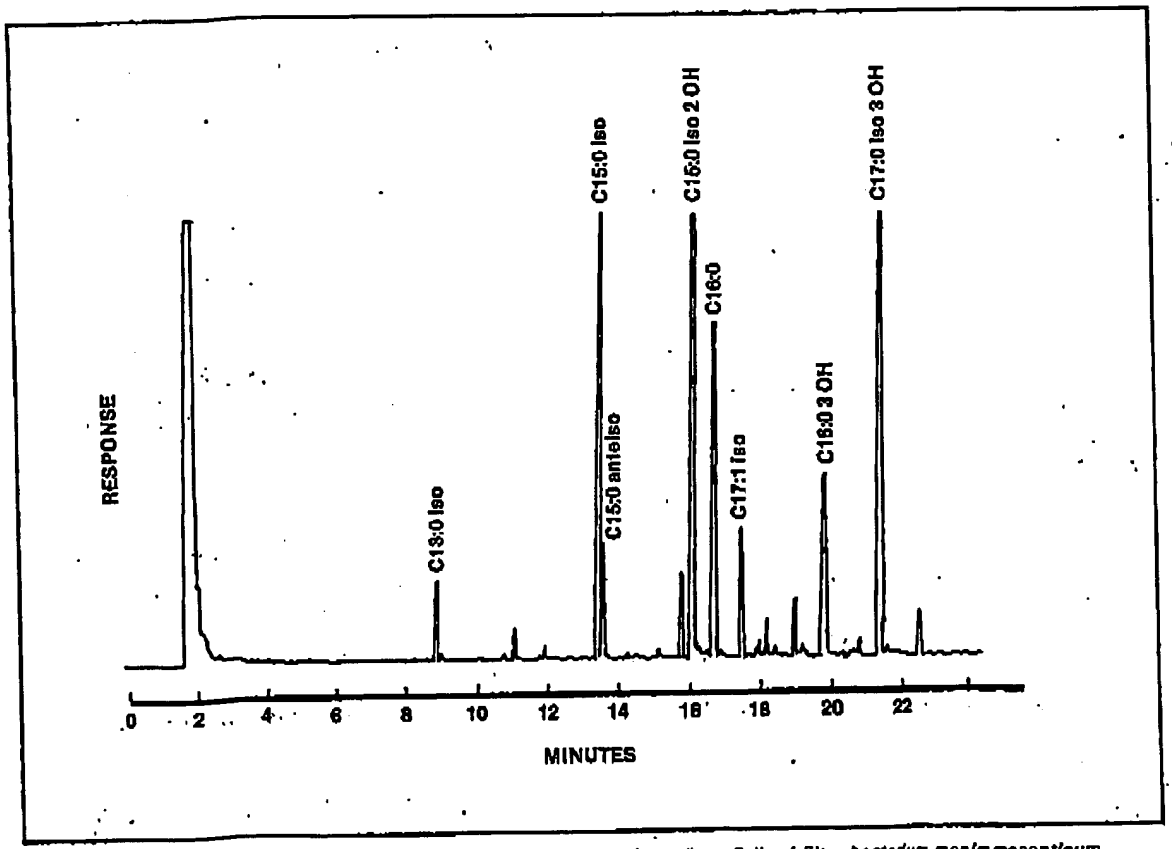


Figure 3. Gas Chromatogram of Fatty Acids (Methyl Esters) from Saponified Whole Cells of *Flavobacterium meningosepticum*

most common fatty acids detected are straight chain fatty acids with each carbon saturated with hydrogen atoms. Mono-unsaturated acids, those having one double bond in the carbon chain, are also common. Di- and tri-unsaturated fatty acids have been isolated in yeast extracts (14). Branching of the carbon chain backbone generally results in "iso" or "anteiso" acids. Branched acids are abundant in Gram positive bacteria such as *Staphylococcus* (Figure 2) and *Bacillus* species. Cyclopropanes, those acids with a three-carbon ring in the carbon chain, are found in many Gram negative organisms. Hydroxy acids have an OH group bonded to the second *(alpha)* or third *(beta)* carbon. (Carbon numbering begins at the carboxylic end of the chain.) These are also commonly found in Gram negative bacteria.

While most fatty acids contain only the carboxyl group, occasionally fatty acids with more than one functional group are isolated. Such acids have been isolated in so few organisms that they are an immediate clue to the identity of the bacterial source. The unique FAME profile of *Flavobacterium meningosepticum*, containing several branched chain hydroxy acids, is shown in Figure 3.

METHODOLOGY

For over 15 years, Dr. C.W. Moss et al., have been using gas chromatography to analyze bacterial fatty acids. The sensitive and reproducible sample preparation technique developed in Dr. Moss's laboratory at the Centers for Disease Control (3,8) is, with a few modifications, schematically represented in Figure 4. The procedure, done in a single test tube which significantly reduces sample loss, is described in detail below.

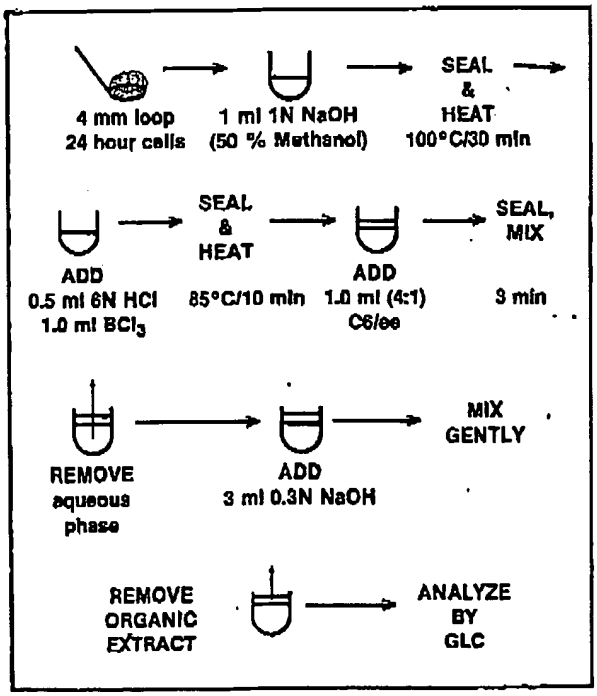


Figure 4.

REAGENTS

1. 1.2N NaOH in 50% aqueous methanol. Dissolve 4.8 grams NaOH (Baker Reagent Grade) in 50 ml distilled water plus 50 ml methanol (Burdick and Jackson). Reagent should be prepared fresh at least every 30 days.
2. 6 *M* HCl. Dilute concentrated (12*M*) HCl (Baker Reagent Grade) one to one with distilled water.
3. 12% BCl_3 in methanol. Supelco, Bellefonte, PA. Available in both pint bottles and 2 ml ampules.
4. Hexane/diethyl ether (1:1) (both Burdick and Jackson).

CULTURE

Using a wire loop, transfer cells from a colony on the primary isolation plate to the center of a plate of basal media, preferably Trypticase Soy Agar (BBL). Moisten a sterile cotton swab with sterile distilled water. Use the swab (or the wire loop) to spread the cells over the entire surface of the plate. Rotate the plate 90° four times while spreading to assure even coverage. Incubate the culture plate at 37°C for 24 hours. (Media selection and incubation conditions may vary for fastidious organisms. However, the culture conditions should be identical to those used to generate the reference profile.) Length and temperature of incubation are important considerations since both factors may influence the fatty acid composition of the cells (3,5). Harvest the cells by gently scrapping the agar surface with a 4 mm inoculating loop. One heaping loopful of cells is an adequate quantity for cellular fatty acid analysis. Some cultures do not adhere well to the wire loop, but these can easily be harvested with the tip of a Pasteur pipette after it has been bent into a loop.

SAMPLE PREPARATION:

1. SAPONIFICATION—Cells are added to 1.0 ml of 1.2N NaOH in 50% aqueous methanol solution in a 100 mm x 13 mm screw cap test tube. The tubes are tightly sealed with Teflon-lined caps, then placed in a boiling water bath for 30 minutes. The tubes should be checked after several minutes. Bubbling in the tubes during saponification indicates leakage.

CAUTION

CAREFULLY TIGHTEN THE CAP OF LEAKING TUBES SINCE HEATING OF SEALED TUBES BUILDS PRESSURE AND CRACKED OR SCRATCHED GLASSWARE COULD BURST. IT IS RECOMMENDED THAT THE HEATING STEPS BE DONE IN A CHEMICAL HOOD; THE WEARING OF SAFETY GLASSES IS ANOTHER SAFETY PRECAUTION.

After 1/2 hour, remove the tubes from the bath and cool to room temperature.

2. METHYLATION—Acidify the saponificate with 0.5 ml 6*M* HCl. Using pH paper, verify that the pH ≤ 2. Add 1.0 ml of 12% BCl_3 in methanol reagent to catalyze the methylation. The BCl_3 methanol reagent will pipette more easily if it is cold (7°C).

Again tightly seal the tube, mix gently, then heat for 5 minutes in a water bath at 85°C. Remove the tube from the water bath and cool to room temperature.

EXTRACTION—Add 1.0 ml of hexane/diethyl ether (1:1) to extract the FAMEs from the aqueous phase. Tightly seal the tubes. Gently but thoroughly mix the tubes end over end for three minutes. (The test tube mixers used in hematology labs work very well.) Allow a few minutes for the phases to separate then pipette and discard the acidified aqueous (bottom) phase.

BASE WASH—Add 3.0 ml of 0.3 N NaOH solution to the organic extract. Cap tightly, then mix gently end over end five times. When the phases separate, use a clean Pasteur pipette to transfer the organic extract (top phase) to a clean sample vial. It may be necessary to centrifuge briefly to break an emulsion. Cap vial to prevent evaporation or spillage.

GAS CHROMATOGRAPHIC INSTRUMENTATION

Hewlett-Packard has developed a capillary Gas-Liquid Chromatography system which has been optimized for analyses such as bacterial fatty acid methyl esters. The HP 5790A Gas Chromatograph has an oven designed for high performance capillary chromatography. Equipped with a flame ionization detector and coupled with an HP 3390A Integrator, this GLC system is capable of precise routine FAME analysis at relatively small costs. The addition of a sampler/event control module HP 18400A and a HP 7671A Automatic Sampler offers automation of the GLC analysis. A HP 85 Personal Computer programmed to do peak identifications can be interfaced into the system.

Capillary chromatography is required so that iso- and anteiso-branched chain acids, as well as positional isomers of unsaturated acids, can be resolved (11). A 50 m x 0.2 mm methyl silicone fused capillary column has both the chromatographic performance and column lifetime desired for routine analysis of bacterial extracts (7, 11). Prior to shipping, every Hewlett-Packard fused silica capillary column is tested to determine column performance. The column recommended for this application (Part No. 19091B Option 102) is required to have ≥ 4000 theoretical plates per meter for peaks with a $k = 7$ to 9, Since the stationary phase has been chemically crosslinked to the fused silica tubing, there is less noise and drift during temperature programmed runs. The crosslinked fused silica capillary columns also are more inert than the packed column systems.

Listed below are the chromatographic conditions suitable for whole cell bacterial FAME analysis with a 50 m methyl silicone fused silica capillary column installed in a HP 5790A Gas Chromatograph.

PNEUMATICS:
- Carrier gas—Hydrogen
- Column Head pressure—25 psi
- Split ratio—100:1
- Septum purge—5 ml/minute

OVEN TEMPERATURES:
- Initial temperature—145°C
- Initial time—0 minutes
- Program rate—5°C/minute
- Final temperature—280°C
- Final time—0 minutes
- Injection port—250°C
- FID temperature—300°C

INTEGRATOR:
- Threshold—1
- Attenuation—2^0
- Peak width—.04
- Option 5 (extended retention time)—ON
- Chart Speed—1 cm/minute

TIME TABLE:
- 0—Int 8 ON
- 0—Area Rej 90000000
- 3.50—Area Rej 0
- 26.00—Stop

INJECT 1 μ

Gas flows to the flame ionization detector should be set as recommended in the HP 5790A Gas Chromatograph User's Manual.

FAME IDENTIFICATION

Before attempting to use GLC to identify bacteria, the operator should be familiar with the use of the instrument. This can be done by performing several injections of a FAME standard solution. On a given day, repeated injections of a standard solution on a HP 5790A GLC system should result in run-to-run retention time differences of less than 0.005 minutes for any peak in the run (15).

A bacterial FAME mix available from Supelco Inc., Bellefonte, PA., contains a selection of methyl esters of the fatty acids commonly found in bacteria (Figure 5). To use the standard with the conditions listed above, dilute 20 microliters of the Supelco standard in 1 microliter of the extraction solvent. The amount injected is 1 microliter. The elution order of the acids in the standard is supplied by Supelco. (Elution order is given for a SP-2100 glass packed column. The order is not altered on the methyl silicone fused silica capillary column. However, isomers of the unsaturated acids can be resolved on a fused silica capillary; two C18:1 peaks may be observed eluting before C18:0.) Notice that the hydroxyl group increases the polarity (boiling point) of hydroxy acids compared to saturated unsubstituted acids so that they elute later than other fatty acids of the same carbon length on a methyl silicone fused silica capillary column. For example, C16:0 2 OH elutes after C17:0 whereas other fatty acids with 16 carbons elute after C15:0 and before C16:0.

The Supelco standard does not contain all the FAMEs needed for bacterial identification. Additional standards containing the iso-branched saturated FAMEs are required. Those fatty acids with two functional groups, such as those in *Flavobacterium meningosepticum* are not commercially available. These acids are best obtained by preparing and derivatizing the acids from live stock cultures of the bacteria. Figure 6 shows chromatograms of a *Xanthomonas maltophilia* and a *Pseudomonas putrefaciens* extract which contain more of these unusual acids. (Identity of these as well as other acids are available in the literature.)

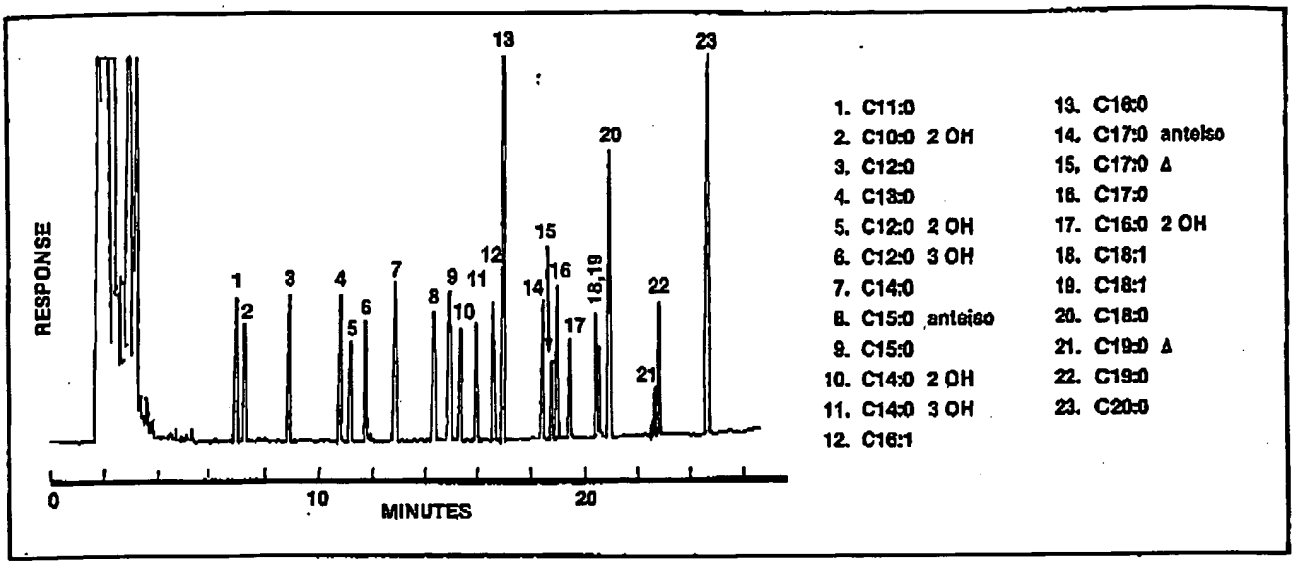


Figure 5. Gas Chromatogram of Bacterial Fatty Acid Methyl Esters Standard Mixture (Supelco)

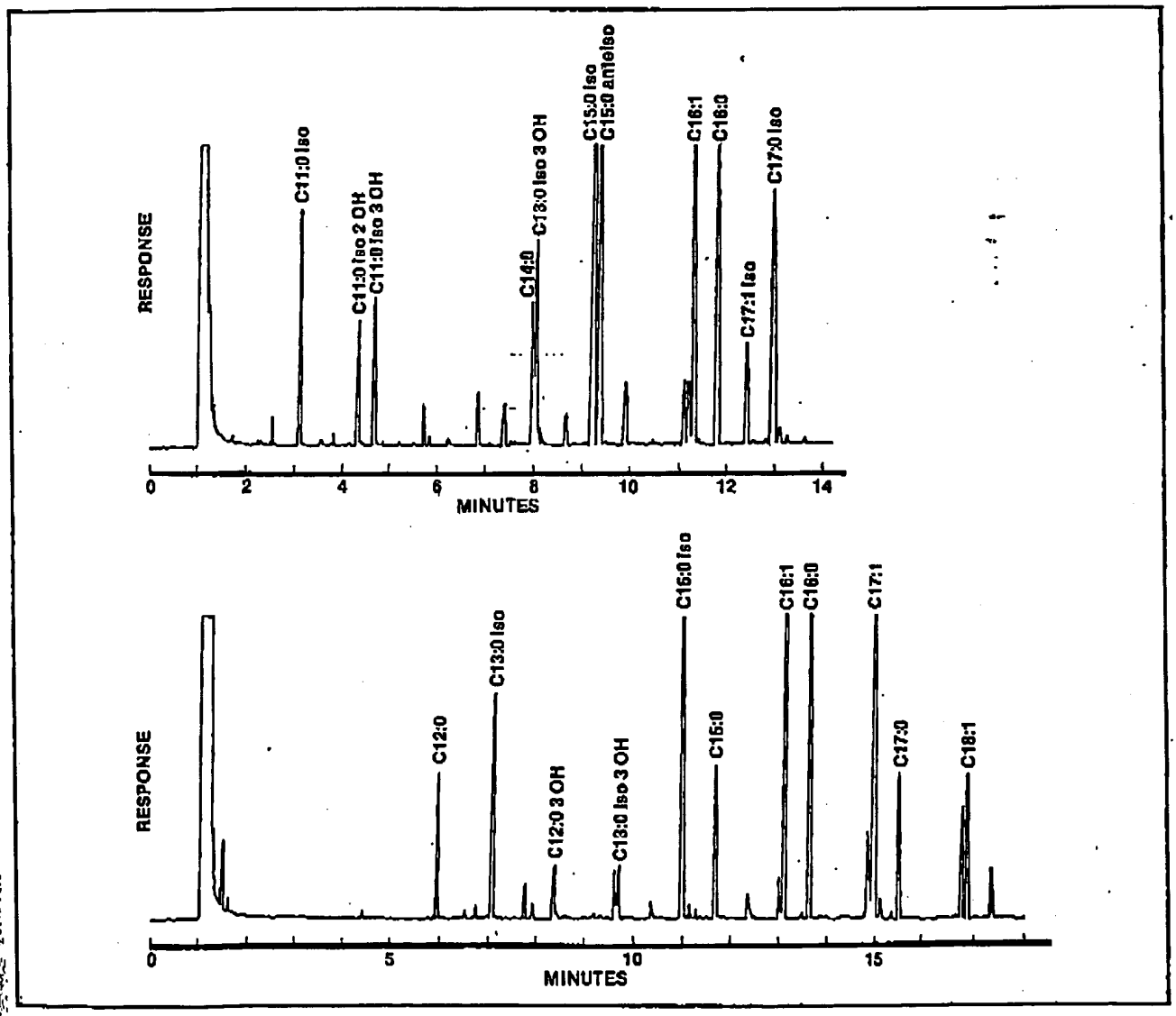


Figure 6. Gas Chromatograms of Methyl Esters of Whole Cell Fatty Acids from *Xanthomonas maltophilia* and *Pseudomonas putrefaciens*

The retention time data obtained while becoming familiar with the instrument can then be used to calculate Equivalent Chain Length (ECL) data for FAMEs. ECL expresses where a FAME elutes with respect to the series of straight chain saturated methyl esters in a temperature programmed run. (This is similar to the better known Retention Index System for hydrocarbons in an isothermal analysis.) The ECL Index of a straight chain saturated FAME is equal to the acid's carbon number. That is, C14:0 has an ECL of 14.000. A FAME eluting half way between C14:0 and C15:0 has an ECL of 14.500.

After identifying the saturated straight chain FAMEs in the Supelco standard, calculate the ECL for the other FAMEs using the following equation, a simple linear interpolation between the saturated straight chain FAMEs:

$$ECL_X = ((Rt_X - Rt_n) / (Rt_{n+1} - Rt_n)) + n$$

where Rt_X is the retention time of the methyl ester x; Rt_n is the retention time of Cn:0, the saturated straight chain methyl ester which precedes methyl ester x; Rt_{n+1} is the retention time of Cn+1:0, the straight chain saturated FAME which elutes after methyl ester x.

Figure 7 is a family plot of ECL data. The ECL for a given FAME is plotted on the vertical axis above Cn:0. Each vertical axis represents the retention time between Cn:0 and Cn+1:0 in ECL units. For members of a homologous series, the ECL plots tend to be linear. This linearity allows one to interpolate from the plot when identifying unknown FAME peaks. (While column-to-column variability is very low for HP fused silica capillary columns coated with methyl silicone, it is recommended that an ECL plot be made for each column.)

Manual identification of FAME peaks is done by calculating the ECL index for each peak. The indices are plotted on the family plot for the specific column. Peaks are identified when they lie within a window of approximately 0.005 ECL units of an ECL value for a known standard FAME (remember that the retention time stability of the system is good to 0.005 minutes; each full ECL unit is equal to roughly 1 minute of retention time with the given GLC conditions).

Retention times of overloaded or very large (offscale) peaks may be distorted. It is advised that the FAME concentration of each extract be comparable to the concentration of the working Supelco standard solution (20 nanograms/1 microliter with a 100:1 split ratio giving 0.2 nanogram on column per microliter injection). You may find it necessary to dilute or concentrate extracts depending on the amount of cellular material available. Extracts can be concentrated by evaporating some of the solvent with a gentle stream of clean nitrogen.

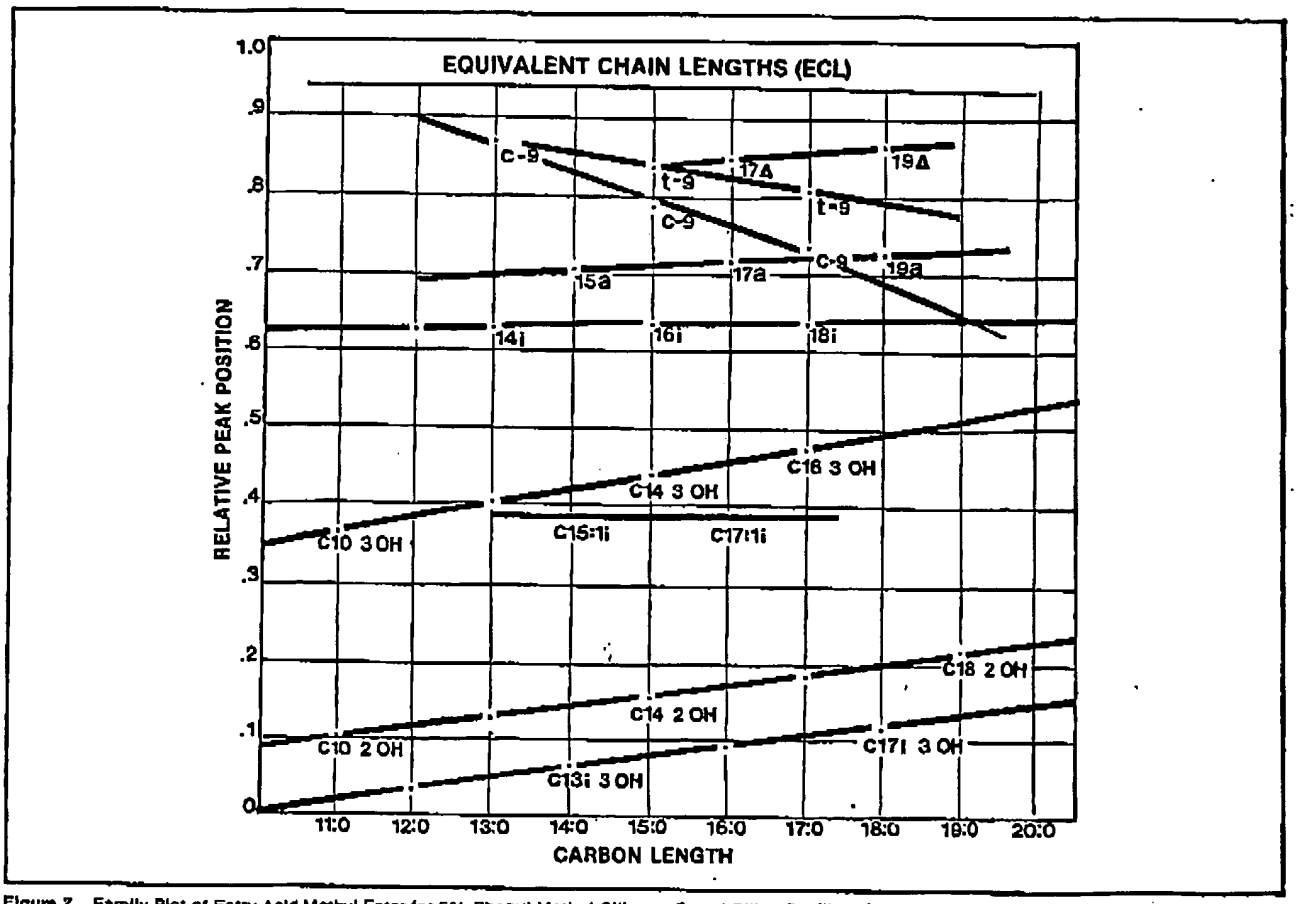


Figure 7. Family Plot of Fatty Acid Methyl Ester for 5% Phenyl Methyl Silicone Fused Silica Capillary Column

Because the instrument is operating at high sensitivity, contamination from various sources can produce peaks which interfere with interpretation. Thus, one should use fresh (less than 30 days) reagents, and prepare a control extract (reagents only) with each batch of samples. Many of the small contaminating peaks appear as small broad peaks with peak widths twice that of the known FAMEs in the run. Eliminate these peaks from the profiles.

When comparing profiles, peaks that are less than 1% of the total area percent should not be considered. Some authors do not consider the presence or absence of small peaks which contribute less than 2% of the total area percent. After eliminating these small peaks, most bacterial extracts will contain 10-12 significant FAMEs. Cultures of the same species will have the same major FAMEs present and in very similar area percent ratios (± 5%). Larger (> 10%) differences in an area percent ratio may indicate a related species.

Software is now available to program the HP 85 Personal Computer for FAME peak identification. For a given column and conditions, ECL values for known bacterial FAMEs are entered into the HP 85. A standard mixture of saturated straight chain FAMEs is injected into the GLC to calibrate the system. Following the calibration run, the HP 85 will match ECL values of peaks in a run with the data in its memory. The HP 85 report lists the ECL, the fatty acid name, and the area percent of each significant peak in the run.

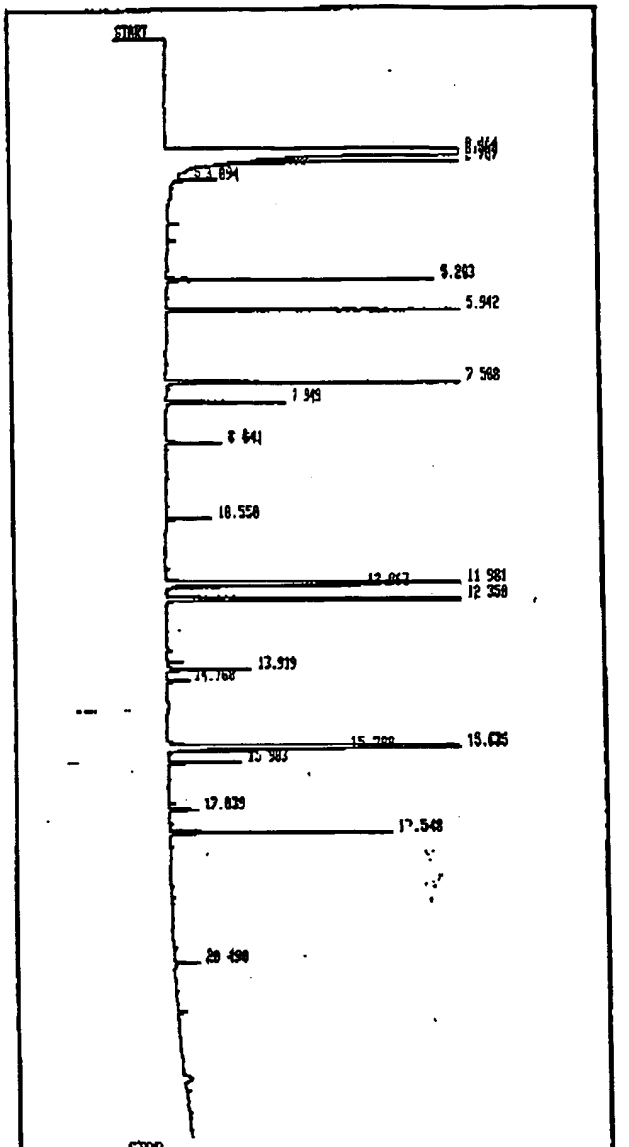
For a sample HP 85 report, see Figure 8. Figure 9 shows the corresponding HP 5790A chromatogram of a *P. aeruginosa* extract, as integrated on a HP 3390A Integrator.

---------- UNKNOWN ----------

RUN # 493 SEP/30/82 12:26:29
BOTTLE # 19

RT	ECL	NAME	AR %
5.28	11.390	C10:0 3OH	2.39
5.94	12.001	C12:0	3.39
7.51	13.146	C12:0 2OH	5.37
7.95	13.429	C12:0 3OH	1.24
11.98	15.798	C16:1 C-9	9.48
12.06	15.843	C16:1 T-9	2.22
12.35	16.003	C16:0	33.53
15.64	17.808	C18:1 T-9	37.63
15.71	17.849	....	2.08
17.55	18.878	C19:0 CYC	2.67

Figure 8. HP 85 Report from Run #493



AREA%

RT	AREA	TYPE	AR/HT	AREA%
2.464	3.2075E+07	TSPB	0.062	99.252
2.565	8566	DTBB	0.010	0.008
2.707	5215	TBP	0.038	0.016
3.094	727	BB	0.025	0.002
5.283	5365	BB	0.027	0.017
5.942	7685	PB	0.028	0.024
7.568	12857	PB	0.038	0.039
7.949	2790	PB	0.031	0.009
8.841	1432	PB	0.034	0.004
18.558	1897	BB	0.033	0.003
11.981	21284	BV	0.033	0.066
12.063	4994	VB	0.034	0.016
12.358	75285	PB	0.034	0.233
13.919	2293	BB	0.037	0.007
14.168	619	PB	0.035	0.002
15.635	84495	PV	0.036	0.262
15.709	4676	VB	0.034	0.015
15.983	1862	BB	0.035	0.006
17.839	907	PB	0.041	0.003
17.548	5995	PB	0.036	0.019
20.498	642	PB	0.035	0.002

TOTAL AREA= 3.2317E+07
MUL FACTOR= 1.0000E+00

Figure 9. Gas Chromatogram of Fatty Acid (Methyl Ester) extract from *Pseudomonas aeruginosa*

SUMMARY

Work done by Dr. C.W. Moss et al., has shown that the analysis of bacterial whole cell fatty acids is a useful and practical technique to rapidly identify bacteria. The procedure developed at CDC is particularly appealing because it is applicable to many different microbial groups. Some species within such diverse groups as *Pseudomonas* and related non-fermenters, *Legionella*, and *Mycobacterium* have been successfully distinguished. The technique is most helpful where traditional microbiological procedures are tedious, time consuming, or ambiguous.

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Lactose, salicin, sucrose, raffinose and glycerol were not attacked.

Strain 3210/64 belonged to O group C_1 and was agglutinated to titer of S. thompson O (6, 7) antiserum and in absorption tests removed all agglutinins from the antiserum.

The flagellar antigens of strain 3210/64 were diphasic. Phase 1 was agglutinated by diagnostic dilutions of z complex antiserum. When tested in v, w, z_{13}, z_{28}, and z_{40} single-factor antisera, agglutination occurred only in antiserum for factor w. The phase 1 antigens of strain 3210/64 were not agglutinated to the full titer of antisera for the first phases of S. worthington (1, w), S. bredeney (1, v), S. westerstede (1, z_{13}), S. javiana (1, z_{28}) and S. rutgers (1, z_{40}), nor did the phase 1 antigens remove all agglutinins from any of those antisera (Table 1).

Phase 2 antigens of strain 3210/64 were agglutinated in diagnostic dilutions of 1, 2; 1, 5; 1, 6; and 1, 7 antisera. When tested with single-factor antisera for antigens 2, 5, 6 and 7, agglutination occurred only in antiserum for factor 2. The organism was flocculated to the titer of S. paratyphi B phase 2 (1, 2) antiserum, and in absorption tests reduced the titer of that antiserum from 1:12,800 to 1:100.

Strain 3210/64 was designated as a new serotype with the antigenic formula 6, 7:l, w:1, 2, and the name Salmonella gabon was proposed for it.

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METHODS FOR CHARACTERIZATION OF STREPTOMYCES SPECIES[1]

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ABSTRACT. The methods used by collaborators in the International Streptomyces Project (ISP) for emendation of descriptions of type and neotype strains of the genus Streptomyces (Actinomycetales) are presented.

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An international cooperative effort, now in progress, is directed toward collection of type cultures of the Streptomyces species for deposition with the Centraalbureau voor Schimmelcultures (CBS), Baarn. From this center the reference cultures will be supplied to other culture collections so that they are available throughout the world.

An essential adjunct to this activity is the redescription of each type culture in terms of currently acceptable criteria and methods. The urgent need for an authentic reference collection, accompanied by standardized characterizations for each species, has been pointed out by spokesmen for the several meetings and conferences which culminated in this project. (See, for example, Gottlieb, 1959, 1961; Küster, 1959; Krasil'nikov, 1961.) More than 40 investigators[2] representing 17 countries are participating in this research. Each culture is described independently by three of these cooperating specialists in different laboratories before it is deposited in the reference collection.

[1] This project is supported in part by a research grant from the National Science Foundation, U.S.A. The Subcommittee on Actinomycetes of the Committee on Taxonomy, A.S.M. and the Subcommittee on Taxonomy of Actinomycetes of the International Committee on Bacteriological Nomenclature are co-sponsoring advisors.

[2] Participants in the 1964-1965 studies are listed on p. 338.

This manual contains the criteria and methods adopted for the project. It reflects the results of two extensive cooperative studies directed toward selection of stable properties and reproducible procedures for characterization of streptomycetes. One study conducted under the direction of the Subcommittee on Actinomycetes of the Committee on Taxonomy, American Society for Microbiology was reported by the Chairman, Dr. D. Gottlieb (1961). A similar preliminary study on an international basis was reported for the Subcommittee on Taxonomy of Actinomycetes of the International Committee on Bacteriological Nomenclature by the Secretary, Dr. E. Küster (1961, 1964). The descriptive criteria are essentially the same as those included in the recommendations of this international subcommittee for descriptions of Actinomycetales appearing in patent applications (Gottlieb, 1963). The methods in mimeographed form have been used successfully for the description of type and neotype strains of more than 200 named species submitted to ISP collaborators during 1964 and 1965, and are now in use for a continuation of the project. Only minor editorial changes have been made except that the test for nitrate reduction (including medium 8, Bacto-nitrate broth) has been omitted. This characteristic proved unstable and has been dropped from the study.

It is hoped that the characterizations used in this manual will be included in future descriptions of Streptomyces species so that direct comparison can be made with descriptions for type cultures in the reference collections.

MATERIALS AND GENERAL METHODS

CULTURE MEDIA

Prepare Difco[3, 4] dehydrated culture media as instructed on labels of containers. If the dehydrated media are not used, use formulas and instructions in this manual as a guide to preparation of the media.

[3] All culture media described in this manual have been prepared especially for the I.S.P. by Difco Laboratories as preformulated dehydrated media. This important contribution by Difco Laboratories is gratefully acknowledged. When Difco dehydrated media are used, instructions on labels supersede instructions in the manual.

[4] Difco Laboratories, Detroit, Michigan, U.S.A. 48201

Sterilize culture media in the autoclave at 121° C. Sterilize loosely packed tubes or flasks containing less than 500 ml for 15 minutes; sterilize larger quantities for 20 minutes. (Do not autoclave carbon compounds to be used in the carbon utilization tests. Special instructions for sterilizing these compounds are given with medium 9.) Adjust pH of media with NaOH or HCl before addition of agar and before sterilization.

Trace salts solution (Use as directed in media 3, 4, 5 and 7 if prepared from formulas. Do not add to the corresponding Difco dehydrated media.)

$FeSO_4 \cdot 7H_2O$	0.1 g
$MnCl_2 \cdot 4H_2O$	0.1 g
$ZnSO_4 \cdot 7H_2O$	0.1 g
Distilled water	100.0 ml

Medium 1: Tryptone-yeast extract broth (Pridham and Gottlieb, 1948)

Bacto-Tryptone (Difco)	5.0 g
Bacto-Yeast Extract (Difco)	3.0 g
Distilled water	1.0 liter

pH 7.0 to 7.2 before autoclaving

Dispense 5 ml of broth into test tubes with a diameter of 20 mm or more. Use these test tubes for initiating growth from lyophile pellet. One tube will be needed for each culture studied.

Dispense 50 ml of the broth into each 250 ml Erlenmeyer flask (or 25 ml into 125 ml flask). These flasks will be used for preparation of washed inoculum (p. 322). One flask will be needed for each culture studied.

Medium 2: Yeast extract—malt extract agar (Pridham et al., 1956-57)

Bacto-Yeast Extract (Difco)	4.0 g
Bacto-Malt Extract (Difco)	10.0 g
Bacto-Dextrose (Difco)	4.0 g
Distilled water	1.0 liter

Adjust to pH 7.3, then add --

Bacto agar	20.0 g

Liquefy agar by steaming at 100° C for 15-20 minutes.

Dispense appropriate amount for a slanting into at least 6 tubes for each culture. Sterilize by autoclaving; cool tubes as slants. Use the agar slants for preparation of stock cultures (page 321).

Also sterilize medium 2 in flasks for pouring the sterilized medium into Petri dishes. Seven standard 90 mm dishes containing 25 ml per plate will be needed for each culture. (Pages 325 and 330).

Medium 3: Oatmeal agar (Küster, 1959a).

Oatmeal	20.0 g
Agar	18.0 g

Cook or steam 20 g oatmeal in 1000 ml distilled water for 20 minutes.

Filter through cheese cloth.

Add distilled water to restore volume of filtrate to 1000 ml.

Add trace salts solution (page 315) . . . 1.0 ml

Adjust to pH 7.2 with NaOH.

Add 18 g agar; liquefy by steaming at 100°C for 15-20 minutes.

Sterlize in flasks for pouring into Petri dishes. Seven standard 90 mm dishes containing 25 ml per dish will be needed for each culture. (Pages 325 and 330)

Swirl medium before pouring to assure even distribution of the oatmeal.

Medium 4: Inorganic salts-starch agar (Küster, 1959a.)

Solution I: Difco soluble starch 10.0 g. Make a paste of the starch with a small amount of cold distilled water and bring to a volume of 500 ml.

Solution II:

K_2HPO_4 (anhydrous basis)	1.0 g
$MgSO_4 \cdot 7H_2O$	1.0 g
NaCl	1.0 g
$(NH_4)_2SO_4$	2.0 g
$CaCO_3$	2.0 g
Distilled water	500 ml
Trace salts solution (p. 315)	1.0 ml

pH should be between 7.0 and 7.4. Do not adjust if it is within this range.

Mix starch suspension and salts solution.

Add agar (Difco) . . . 20.0 g

Liquefy agar by steaming at 100°C for 15-20 minutes.

Sterilize in flasks for pouring into Petri dishes. Seven standard 90 mm dishes containing 25 ml per dish will be needed for each culture. (Pages 325 and 330)

Medium 5: Glycerol-asparagine agar (Pridham and Lyons, 1961)

L-asparagine (anhydrous basis)	1.0 g
Glycerol	10.0 g
K_2HPO_4 (anhydrous basis)	1.0 g
Distilled water	1.0 liter
Trace salts solution (page 315)	1.0 ml

The pH of this solution is about 7.0-7.4. Do not adjust if it is within this range.

Agar . . . 20.0 g

Liquefy agar by steaming at 100°C for 15-20 minutes.

Sterlize in flasks for pouring into Petri dishes. Seven standard 90 mm dishes containing 25 ml per dish

will be needed for each culture. (Pages 325 and 330)

The final pH of the medium after sterilization with agar and solidification is about 7.4.

Medium 6: Peptone-yeast extract iron agar (Tresner and Danga, 1958)

Bacto-Peptone Iron Agar, dehydrated (Difco)	36.0 g
Bacto-Yeast Extract (Difco)	1.0 g
Distilled water	1.0 liter

pH should b 7.0-7.2 before autoclaving; adjust if necessary.

Liquefy agar by steaming at 100°C for 15-20 minutes.

Dispense appropriate amount for slanting into 2 tubes for each culture. Sterilize and solidify as slants. (Page 334). (Note that less than 1 liter of this medium is easily prepared by using a proportionately smaller amount of the dehydrated peptone iron agar and adding yeast extract in proportion of 0.1% of water used.)

Bacto-Peptone Iron Agar, dehydrated, contains the following ingredients when reconstituted as 36.58 grams per liter of water: Bacto-Peptone, 15 g; Proteose Peptone, Difco, 5 g; Ferric Ammonium Citrate, 0.5 g; Dipotassium Phosphate, 1 g; Sodium Thiosulfate, 0.08 g: Bacto-Agar, 15 g.

Medium 7: Tyrosine agar (Shinobu, 1958)

Glycerol	15.0 g
L-tyrosine (Difco)	0.5 g
L-asparagine (Difco)	1.0 g
K_2HPO_4 (anhydrous basis)	0.5 g
$MgSO_4 \cdot 7H_2O$	0.5 g
NaCl	0.5 g
$FeSO_4 \cdot 7H_2O$	0.01 g
Distilled water	1.0 liter
Trace salts solution (page 315)	1.0 ml

Adjust to pH 7.2-7.4

Bacto-Agar	20.0 g

Liquefy by steaming at 100°C for 15-20 minutes.

Dispense appropriate amount for slanting into 2 tubes for each culture; sterilize and solidify as slants. (Page 333).

Medium 8: Nitrate broth (Deleted because of unreliability of the nitrate-reduction test).

Medium 9: Carbon utilization medium (Modified from Pridham and Gottlieb, 1948)

A. Sterile carbon sources

Use chemically pure carbon sources certified to be free of admixture with other carbohydrates or contaminating materials. Carbon sources for this test are:

No carbon source (negative control)
D-glucose (positive control)
L-arabinose

Sucrose	D-fructose
D-xylose	Rhamnose
I-inositol	Raffinose
D-mannitol	Cellulose

Sterilize without heat by one of the following methods:

1. Filtration. Filter sterilize 10% solution through bacteriological filter. (i-Inositol and cellulose are not sufficiently soluble for sterilization by this method—use one of the methods described below.)

2. Ether sterilization. Weigh an appropriate amount of the dry carbon source and spread as a shallow layer in a pre-sterilized Erlenmeyer flask fitted with a loose cotton plug. Add sufficient acetone-free ethyl ether $(C_2H_5)_2O$ to cover the carbohydrate. (OBSERVE PRECAUTIONS AGAINST FIRE! Allow ether to evaporate at room temperature under a ventilated fume hood overnight or longer. When all ether has evaporated add sterile distilled water aseptically to make a 10% w/v solution of the carbon source.

3. Ethylene oxide sterilization. (Judge and Pelczar, 1955). Make a 10% w/v solution of the carbon source. Cool the liquid in an ice bath to 3-5°C and add 1 volume per cent liquid (cold) ethylene oxide with a chilled pipette or syringe. Agitate the solution. Leave it in the cold ice bath under a ventilated fume hood for

1 hour. Transfer to a warm water bath (about 45°C) UNDER FUME HOOD to permit complete volitilization of the ethylene oxide. The vapors are toxic and explosive.

Carbon sources sterilized by one of these three methods will be added to the basal mineral salts agar to give a final concentration of 1%. For example, add 10 ml of 10% solution to 100 ml basal medium, or 100 ml of a 10% solution to 1000 ml basal medium.

B. Pridham and Gottlieb trace salts (only 1 ml of this solution is used per liter of final medium)

$CuSO_4 \cdot 5H_2O$	0.64 g
$FeSO_4 \cdot 7H_2O$	0.11 g
$MnCl_2 \cdot 4H_2O$	0.79 g
$ZnSO_4 \cdot 7H_2O$	0.15 g
Distilled water	100.0 ml

Store at 3-5°C until required for use. Bring to room temperature before using. Prepare fresh solution each month. Disregard any precipitate or scale (probably iron salts) that forms during storage. (Only 1 ml of this solution will be used in the medium.)

C. Basal mineral salts agar (use analytical reagent grade chemicals)

$(NH_4)_2SO_4$	2.64 g
KH_2PO_4 anhydrous	2.38 g
$K_2HPO_4 \cdot 3H_2O$	5.65 g
$MgSO_4 \cdot 7H_2O$	1.00 g
Pridham and Gottlieb trace salts (B)	1.00 ml
Distilled water	1.00 liter

Dissolve ingredients and check pH. Adjust, if necessary, to 6.8-7.0 with 1 N NaOH or 1 N HCl.

Add agar (*Difco*)	15.0 g

D. Complete medium

Sterilize basal agar medium (C); cool it to 60°C and add sterile carbon source (A) aseptically to give a concentration of approximately 1%. Agitate the mixture and pour 25 ml of medium per dish into 9 cm Petri dishes. Each organism will require 2 Petri dishes with no carbon (as a negative control) plus duplicate plates for each carbon source tested. (Page 335)

METHODS FOR INITIATING GROWTH AND PREPARING STOCK CULTURES FROM LYOPHILE PELLET

1. Make a file scratch on ampoule at a location near upper part of looped cord (see Fig. 1).
2. Immerse the unbroken ampoule into 70% ethyl alcohol.
3. Enclose the alcohol-moistened ampoule with a piece of sterile cotton or cotton and gauze; then snap or break it at the file scratch (Fig. 2).
4. Use sterile forceps or a sterile stiff wire hook to transfer the looped string and pellet to the labeled tube containing 5 ml of sterile tryptone—yeast extract broth (Fig. 3). If a pellet fails to come out attached to the string, the cells or spores on the string will usually be adequate to start a good culture.
5. Shake the tube by hand until the pellet dissolves.
6. Incubate the tubes in a slanted position or on a mechanical shaker to give good aeration. Use of a shaker is the preferred method. Incubate at 25-28°C for 24-28 hours (or until there is evidence of spore germination or growth).
7. Look for possible contaminants with the microscope. Also streak one loopful of the broth culture onto the agar surface of a Petri dish containing medium 2 (yeast extract—malt extract agar). This plate can be examined after a few days to confirm absence of contaminants.
8. Inoculate 6 or more test tube slants of medium 2 (yeast extract—malt extract agar) and of medium 3 (oatmeal agar) with 0.1-0.2 ml of the 24-48 hour growth. Streak material over entire surface of agar slant.
9. Incubate the slants at 25-28°C for 14 days to get mature stock cultures for use in preparation of inoculum (see section which follows). Then store stock slants in refrigerator (6-10°C) until ready for use. Generally stock cultures for preparation of inoculum for characterization tests should be used within one month.

PREPARATION OF INOCULUM

Use stock culture slants (prepared as described in the preceding section) for preparation of (A) general inoculum for all inoculations except carbon utilization test, and (B) a special washed inoculum for determining carbon utilization patterns.

A. Preparation of general inoculum

1. Prepare a supply of stoppered test tubes containing 3-5 ml of sterile distilled water.

2. Use a wire loop and standard aseptic technique to transfer spores, or mycelial growth, from a stock culture slant to one of the tubes of sterile distilled water.

   a. If sporulation on the stock slant is good, transfer sufficient spore material to make a very turbid suspension in the distilled water. Normally most of the spore surface from a stock slant will be required. If necessary, use more than one slant to get a turbid suspension.

   b. If spores are not formed, use the wire loop to transfer mycelial material to the tube of sterile distilled water. Triturate the mycelium in the distilled water with a sterile glass rod or the tip of a sterile pipette. Produce a very turbid suspension of mycelial fragments. Do not use a mycelial suspension if a good spore suspension (a) can be obtained.

3. This distilled water suspension of spores or mycelial fragments may be used immediately as general inoculum or may be held at room temperature 3-4 hours. Prepare fresh inoculum suspensions for tests performed on different days.

B. Preparation of washed inoculum

1. Prepare 5 ml of turbid suspension of spores or mycelium in sterile water as described for general inoculum.

2. Transfer 4-5 ml of this suspension to 50 ml of medium 1 (tryptone—yeast extract broth) in a 250 ml Erlenmeyer flask (or 25 ml in a 125 ml flask).

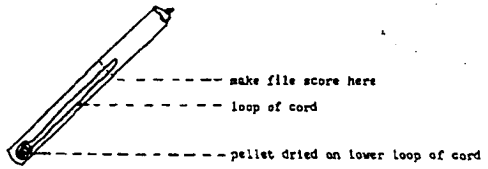

Figure 1: Sealed evacuated ampoule containing lyophile pellet and cord

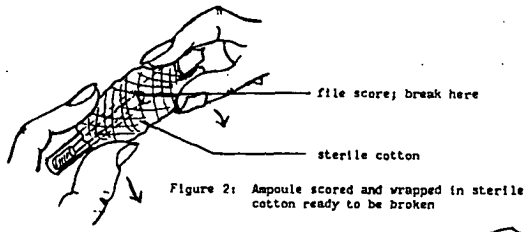

Figure 2: Ampoule scored and wrapped in sterile cotton ready to be broken

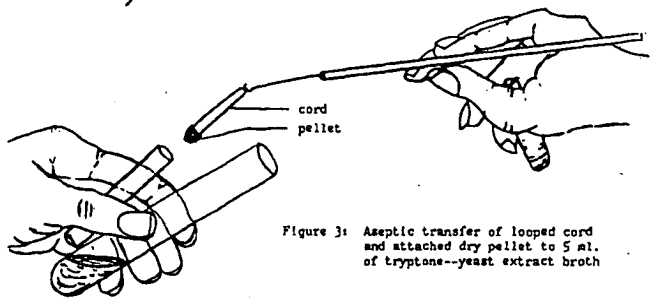

Figure 3: Aseptic transfer of looped cord and attached dry pellet to 5 ml. of tryptone--yeast extract broth

3. Incubate the flask for 48 hours at 25-28°C. If possible the flask should be aerated on a mechanical shaker during the incubation.

4. Use vigorous agitation with sterile glass beads, a mechanical blender such as the Waring Blendor, or other appropriate means to break up the 48-hour growth.

5. Transfer 5-10 ml of this fragmented broth culture into sterile centrifuge tubes equipped with sterile caps.

6. Centrifuge the suspension.

7. Decant the supernatant broth. Add sterile distilled water (or sterile 0.85% NaCl) to restore the original volume in the centrifuge tube. Mix and resuspend the washed sediment with a sterile rod or pipette.

8. Repeat steps 6 and 7. (Compare amount of sediment from different cultures. When amount of sediment is much less than normal amount, use proportionately less water for the final resuspension.)

9. Use the resuspended inoculum at once or within 3 hours to inoculate carbon utilization tests.

PROCEDURES FOR CHARACTERIZATION OF CULTURES

I. MORPHOLOGICAL CHARACTERIZATIONS

Accurate morphological characterization of the Actinomycetes producing catenulate spores is obviously dependent upon use of a culture medium giving good sporulation. Four media which gave good performance in this respect in previous cooperative studies are listed below as "standard" media. If these four media all fail to give good development of the sporulating aerial mycelium, then an additional medium promoting good spore formation should be used.[5] If it is necessary to use an additional medium, also include a record of observations of the growth on the standard media. Spore chain and sporophore morphology should be determined by observation of a fully matured culture with good

[5] Cooperators in the ISP will please communicate the formula of any additional medium used to Dr. Shirling, who will then forward the formula to the other two cooperating investigators studying the culture.

spore formation. Do not use a culture in which degeneration through autolysis, hygroscopic properties, or extreme dehydration may have altered the morphology.

A. Culture media for morphological studies

1. The "standard" culture media for morphological studies for all cultures will be medium 2 (yeast extract—malt extract agar); medium 3 (oatmeal agar); medium 4 (inorganic salts—starch agar); and medium 5 (glycerol—asparagine agar).

2. Pour 7 plates of each medium using 25 ml of medium in each 9 cm diameter Petri dish.

   Media should be cooled to about 50°C and dispensed aseptically into sterile Petri dishes. Poured media should be held for a minimum of 24 hours at 25-28°C to promote moderate drying and to check sterility before inoculation.

B. Inoculation of plates for morphological studies

1. Use general inoculum prepared according to directions on page 322.

2. Place about 0.05 ml of the inoculum onto the agar surface near one edge of the Petri dish (1 drop from a 1 ml serological pipette). This drop will serve as a "pool" of inoculum.

3. Use a flame-sterilized wire loop to make 5 equally-spaced streaks across the plate, dipping the loop into the pool of inoculum prior to each streak (see Fig. 4).

4. Make crosshatch streaks as shown in Fig. 5.

5. Inoculate a given culture onto at least 7 Petri dishes for each medium.

6. Incubate plates in the dark at 25-28°C. For each culture observe two plates of each medium after 7 days, two at the end of 14 days, and two at the end of 21 days. One extra plate is inoculated in case of accident.

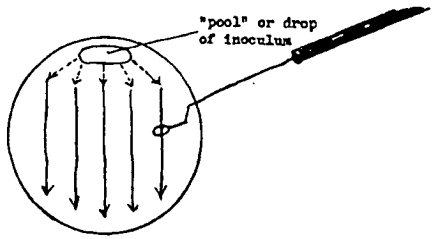

Figure 4: Five initial streaks; loop dipped into inoculum for each streak

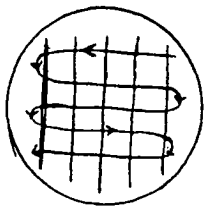

Figure 5: Cross streaks made without picking up additional inoculum

C. Determination of micromorphological characteristics of the spore-bearing hyphae

1. Determine the characteristics of the spore-bearing hyphae and spore chains by direct microscopic examination of the culture surface on opened dishes of the crosshatched cultures. Use a magnification adequate to establish the presence (or absence) of chains of spores. This will usually be 100x - 700x. A minimum of 10 microscope fields at 100x should be examined.

2. Determine the number of spores at the end of mature hyphae. State whether spores occur (a) singly, (b) in pairs, (c) in chains of 3-10, or (d) in chains of more than 10. If there is variation state the range of variation and then choose the most representative spore number (a, b, c, or d) for the culture on a given medium. Make the observation on each medium which shows good sporulation.

3. The form of the spore chain and spore-bearing hyphae should be described only with mature cultures on which many well-defined spore chains can be seen. Describe sporulating cultures in terms of the morphological groups of Pridham et al. (1958), modified according to Baldacci (see Figs. 6-13). In some cases, particularly with cultures producing open loops and

primitive spirals, more than one morphological type may be observed. On the basis of extensive observation of the culture surface, record all mature sporophore-spore chain types seen with an estimate of the percent of spore-bearing aerial hyphae falling into each category.

SIMPLE	VERTICILLATE
Straight - Rectus (R)	Monoverticillus (MV)
Flexible - Flexibilis (F)	Monoverticillus-Spira (MV-S)
Open loops - Retinaculum-Apertum (RA)	Biverticillus (BIV)
Spirals - Spira (S)	Biverticillus-Spira (BIV-S)

Undetermined (U)

4. Photograph typical morphology of the sporulating hyphae, or make simple drawings.

5. Retinaculum-Apertum: Record the approximate diameter of characteristic open loops and primitive spirals of RA cultures.

6. Verticillate: (a) Record average diameter of the main hyphae from which verticils originate. (b) Record whether or not verticils are evenly spaced along the main hyphae.

D. Special morphological observations

It is also important to look for the following characteristics (usually identified with other actinomycete groups):

1. Presence of globular sporangia as in Actinoplanaceae

2. Presence of flagellated spores, as in Actinoplanes

3. Formation of conidia-like spores on the substrate hyphae

4. Tendency of the substrate mycelium to fragment

5. Occurrence of sclerotia.

SIMPLE

Figure 6. Rectus (R)
or straight

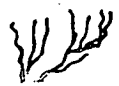

Figure 7. Flexibilis (F)
or flexuous

Figure 8.
Retinaculum-Apertus (RA)
Open loops, hooks, or extended spirals of wide diameter

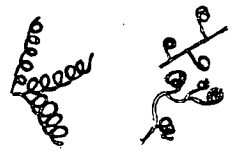

Figure 9. Spira (S)
Simple spirals (not on verticils). Spirals may be short and compact or long, extended, or open

VERTICILLATE

True verticillates have main axis of wider diameter than branching hyphae, and have uniform spacing between verticils.

Figure 10. Monoverticillus (MV)
Primary verticils or whorls distributed on a long axis or branch; no spirals

Figure 11.
Monoverticillus-Spira (MV-S)
Primary verticils or whorls distributed on a long axis; elements of verticils spiralled

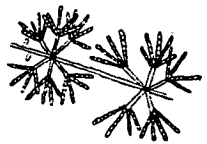

Figure 12. Biverticillus (BIV)
Compound verticils or whorls on a long axis; no spirals

Figure 13.
Biverticillus-Spira (BIV-S)
Compound verticils; elements of secondary verticils spiralled

Figures 6-13. Reproduced from Pridham et al. (1958) with permission of authors and publisher.

E. Spore morphology and surface

Laboratories having access to an electron microscope should include electron micrographs of the spore surface as one of the descriptive characterizations for each type culture. Electron micrographs should also show spore chains. Suggested method:

1. Use the same crosshatch Petri dish cultures prepared for observation under the light microscope.

2. Electron microscope specimen grids coated with Formvar or collodion are gently pressed to the aerial surface of a culture with mature spores.

3. Spore chains which adhere to the coated surface of the grids are observed and photographed in the electron microscope without fixing or shadowing at a magnification of 8,000x - 10,000x.

4. Preserve the picture record to include with the report. In addition to the photographic record, spore silhouettes should be characterized as smooth, spiny, hairy, warty (see Fig. 14).

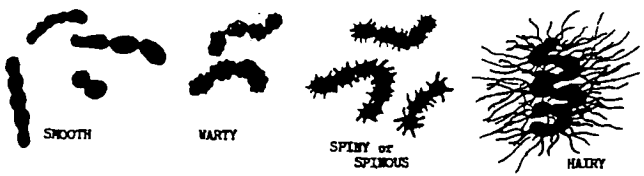

Figure 14.

II. COLOR DETERMINATIONS

Color determinations should be made for (1) the mass color of mature, sporulating aerial surface growth, (2) for the color of the substrate mycelium as viewed from the reverse side, and (3) for diffusible soluble pigments other than melanins. The Tresner-Backus color series, adopted by participants in the 1962 Montreal Workshop (Küster, 1964; Tresner and Backus, 1963) is specified for spore mass color determinations. In this system, seven color series

are delineated by color wheels made with tabs from the 3rd or 4th edition of the Color Harmony Manual (Jacobson et al. 1948; Eckerstrom and Foss, 1958).[6] The Tresner-Backus color guide is not designed for determination of substrate mycelium colors or colors of soluble pigments. Although a color guide is not required for these colors, the substrate mycelium color may be identified in terms of a special guide[7] prepared for the I.S.P. by H. Prauser (1964) or in terms of the Bondartsev (1954) color scale.

A. Culture media for color determinations

Use the following media: Medium 2 (yeast extract—malt extract agar); medium 3 (oatmeal agar); medium 4 (inorganic salts—starch agar); and medium 5 (glycerol—asparagine agar). The crosshatch plates that were prepared for morphological studies may be used for this purpose.

B. Observations

1. Observe plates at 7, 14 and 21 days as prescribed for morphological studies. Color determinations should be recorded only for mature cultures.

2. Use north-window daylight on a bright day. If this is not possible, use a balanced and reproducible system of artificial light simulating north sky daylight.

C. Determination of aerial mass color

1. Observations should be limited to mature cultures with a heavy spore mass surface.

[6] A complete set of Tresner-Backus color guides has been provided to each participant in the I.S.P. Printed patterns for assembling the color wheels, together with reprints of the Tresner-Backus paper can be obtained without cost from Prof. E. B. Shirling, I.S.P., Ohio Wesleyan University, Delaware, Ohio 43015, U.S.A. Color Harmony Manual chips used in the guide may be purchased from Color Standards Department, Container Corporation of America, 38 South Dearborn St., Chicago, Illinois 60603, U.S.A.

[7] Prepared for I.S.P. participants by H. Prauser (1964) from color tabs of Baumanns Farbtonkarte Atlas I.

2. Determine the Tresner-Backus color series (Red, Yellow, Green, Blue, Violet, Gray or White) by comparing mature spore mass color on all appropriate media with the Tresner-Backus color wheels. Use only the mat (dull) surface of the color tabs. Most cultures will fall within the range delineated by one wheel. There are a few instances in which color tabs assigned to two different wheels are similar in color. If a culture is intermediate between these two tabs from different wheels, it may be difficult to choose one series as better than the other for describing the culture. In these rare cases classify the culture in both series.

3. Identify by number-letter code (printed on the tab), the one color tab which most nearly characterizes the spore-mass color. Also record the ISCC-NBS (U.S. Dept. of Commerce, 1955) name listed in the color wheel folder for the tab chosen to characterize the spore-mass color. If the color is intermediate between two tabs, designate both.

D. Color of substrate mycelium (reverse color)

The color of the substrate mycelium is determined by observing the reverse (under) side of mass growth on the various media. A method for removing most of the agar medium is described below. A simple guide for use in cutting off the agar is provided.

1. Place the cutting guide onto layers of filter paper or paper towel saturated with wet disinfectant solution (see Fig. 16). (Also provide a beaker of disinfectant for discarding work tools.)

2. Use a cork borer (or similar sharpened cylinder) with a diameter slightly less than the hole in the template to cut an agar plug from typical mature mycelial growth on a Petri dish culture (see Fig. 15). Determine color for (a) mature substrate mycelium and (b) the relatively young edges of the spreading growth.

3. Push the plug from the cylinder with a glass rod so that it turns over and drops inverted onto the cutting guide (Fig. 16). Push the inverted plug into the hole in the cutting guide.

4. Slide a razor blade across the guide slicing off all agar medium which extends above the level of the surface (Fig. 17).

5. It is now possible to observe the reverse side of the culture plug remaining in the cutting guide. Very little agar will remain to interfere with color determination.

6. Assign the culture to one of the following color groups (Szabó and Marton, 1964)
   - yellow-brown
   - yellow-brown + red (or orange)
   - yellow-brown + blue or violet
   - yellow-brown + green

7. In addition, if either Prauser or the Bondartsev color code or the complete Color Harmony Manual is available, identify the color with the nearest matching panel on one of these color guides.

8. Determine the response of color of substrate mycelium to pH change by adding a drop or more of 0.05 N NaOH and 0.05 N HCl to the specimen prepared as described in steps 1-4 above. Make observations immediately and after 10 to 15 minutes.

E. Soluble colors other than melanoid pigmentation

If soluble colors other than brown or black are produced on any medium (or if brown is distinctly modified with red, yellow, green, blue, or violet), 1) record the medium; 2) record the color as the simple unmodified color name (red, orange, yellow, green, blue, violet—use no other color terminology); and 3) in every case of soluble color other than melanoid pigment, determine response of color to pH change by addition of a drop or more of 0.05 N NaOH and 0.05 N HCl to the colored agar. Record whether or not soluble color is affected by pH change. Make observations immediately and after 10 to 15 minutes.

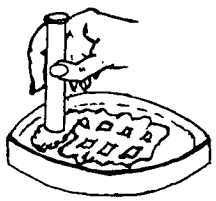

Figure 15: Cut agar plug from a broad mass of mature growth.

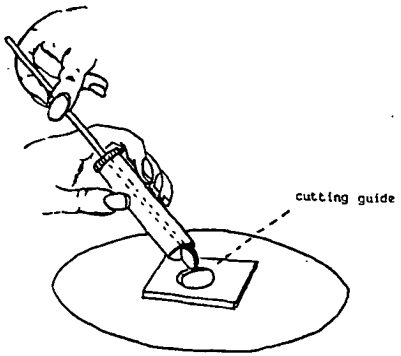

Figure 16: Push plug so that it drops inverted into the cutting guide.

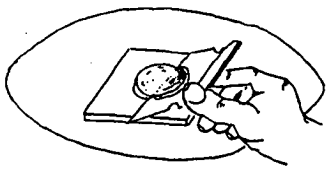

Figure 17: Use razor blade to cut off and discard all agar which extends above the surface of the cutting guide. Observe the exposed reverse side of the mycelium which remains in the cutting guide.

III. PHYSIOLOGICAL CHARACTERISTICS

A. MELANIN PRODUCTION

1. Culture media and inoculation for melanin production

Determine production of melanoid pigments on agar slants of medium 6 (peptone iron agar, Difco, supplemented with 0.1% yeast extract), and medium 7 (Shinobu's modification of Masumoto's tyrosine agar). Also observe other organic media, especially medium 1 (tryptone—yeast extract broth, used for obtaining growth from lyophile pellet).

Use agar slant cultures prepared as described under MATERIALS AND GENERAL METHODS as inoculum for media 6 and 7. Cultures used as inoculum source should be less than 3 weeks old (except for cultures unusually slow in producing mature aerial growth). Use a heavy inoculum of spores and aerial mycelium picked up on a standard wire loop. Streak this inoculum up the surface of the agar slant. Inoculate each experimental culture onto 2 slants of medium 6, and 2 slants of medium 7.

2. Observations and interpretations

a. Observe melanoid pigments on medium 6 and medium 7 after 2 days and after 4 days. Compare inoculated tubes with uninoculated controls. Cultures forming a greenish brown to brown to black diffusible pigment or a distinct brown pigment modified by other color shall be recorded as positive (+). Absence of brown to black colors, or total absence of diffusible pigment, shall be recorded as negative (-) for melanoid pigment production.

b. Other organic media containing peptones or tyrosine, including medium 1 (tryptone—yeast extract broth) used for initial growth from the lyophile pellet, should be observed for production of melanoid pigments. The presence of pigments in these media should be included in the record.

B. CARBON UTILIZATION

1. Basal medium and carbon sources

Detailed instructions for medium 9 (Pridham and Gottlieb carbon utilization medium) including carbon source sterilization are given on pages 318-319.

After autoclaving the basal agar medium, cool it to 60°C and add sterile carbon source aseptically to give a concentration of approximately 1%. Agitate the mixture and pour 25 ml of medium per dish into 9 cm Petri dishes. Prepare duplicate plates of each carbon compound for each culture to be tested. Store medium in refrigerator.

Carbon sources and controls required for the test are repeated below:

No carbon source (negative control)
D-glucose (positive control)
L-arabinose

Sucrose	D-fructose
D-xylose	Rhamnose
I-inositol	Raffinose
D-mannitol	Cellulose

2. Procedures for carbon utilization tests

a. Prepare a washed inoculum as described on pages 323-324.

b. Dry the uninoculated plates by leaving them at room temperature for 4 hours after they are freshly poured or after removing them from refrigerator storage.

c. Place approximately 0.05 ml of washed inoculum (1 drop from a sterile 1 ml serological pipette or dropping pipette) onto one edge of the agar surface. Streak the drop straight across the dish (see Fig. 18). Repeat with a second drop. Inoculate duplicate plates. Use only one culture per plate to avoid false positives due to cross feeding.

d. Observe plates at 10-16 days. Always compare growth on a given carbon source with the two controls; growth on basal medium alone, and growth on basal medium plus glucose.

e. Record results as follows:

Strongly positive utilization (++), when growth on tested carbon in basal medium is equal to or greater than growth on basal medium plus glucose.

Positive utilization (+), when growth on tested carbon is significantly better than on basal medium without carbon, but somewhat less than on glucose.

Utilization doubtful (±), when growth on tested carbon is only slightly better than on the basal medium without carbon and significantly less than with glucose.

Utilization negative (-), when growth is similar to or less than growth on basal medium without carbon. (Always record utilization as negative if growth is not better than no-carbon control.)

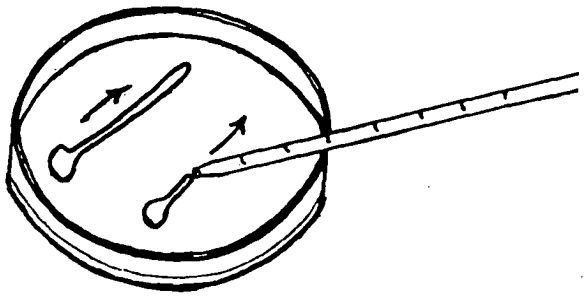

Figure 18.

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PROPOSED EMENDATIONS OF THE INTERNATIONAL CODE OF NOMENCLATURE OF BACTERIA AND VIRUSES — WITH COMMENTS

Compiled by the Editorial Board of the Judicial Commission of the International Committee on Bacteriological Nomenclature - IAMS

Item 1. The title of this Code is "The International Code of Nomenclature of Bacteria."

Comment: The present title of the Code is International Code of Nomenclature of Bacteria and Viruses. The present title was adopted in 1953 at the Rome International Microbiological Congress. By action of the Executive Committee of the International Association of Microbiological Societies (IAMS), the International Subcommittee on the Nomenclature of Viruses has been replaced by a new committee coordinate with the International Committee on Bacteriological Nomenclature. This committee will be constituted at the 1966 IXth Congress for Microbiology in Moscow. It is anticipated that this new International Committee will formulate a Code of Nomenclature for the Viruses. It is no longer appropriate that the name "viruses" be included in the name of the Bacteriological Code.

Item 2. The word virus (or viruses) will be deleted from the bacteriological code wherever there is implicit the assumption that nomenclature of the viruses is included.

Comment: The deletions have been approved by Sir Christopher Andrewes, formerly chairman of the International Subcommittee for Nomenclature of Viruses and currently chairman of the Provisional Committee on Viruses which is to report at Moscow. The deletions will be noted in subsequent Items where pertinent.

Item 3. Emendation of General Consideration 1 (page 3) to read as follows:

"The progress of bacteriology can be furthered by a precise system of nomenclature which is properly integrated with the systems used by botanists, zoologists and virologists, and accepted by the majority of bacteriologists in all nations. The International Code of Nomenclature of Bacteria applies to bacteria and related organisms. Botanical, zoo-

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Comparison of the Cellular Fatty Acid Composition of a Bacterium Isolated from a Human and Alleged To Be *Bacillus sphaericus* with That of *Bacillus sphaericus* Isolated from a Mosquito Larvicide

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The cellular fatty acid (CFA) composition of the cytoplasmic membrane of a bacillus isolated from a human lung and deposited in the National Collection of Type Cultures as *Bacillus sphaericus* NCTC 11025 was determined by gas-liquid chromatography. The CFA composition of *B. sphaericus* 2362, isolated from a microbial larvicide, and those of *B. sphaericus* reference strains obtained from public collections were also determined. Samples were grouped by hierarchical cluster analysis based on the unpaired-group method using arithmetic averages. Samples that linked at a Euclidean distance of ≤2.0 U were considered to belong to the same strain. NCTC 11025 and the type strain of *B. sphaericus*, ATCC 14577, were mixed; all other isolates were monotypic. The predominant fatty acid in NCTC 11025 was 12-methyltetradecanoic acid, while the predominant fatty acid in the remaining isolates was 13-methyltetradecanoic acid. NCTC 11025 linked to the other isolates at a Euclidean distance of 83.8 U, and we concluded that it belongs to a different species that we could not identify. We could distinguish among six DNA homology groups of *B. sphaericus* by using fatty acids. Within DNA homology group IIA, strain 2362 could be distinguished from other strains belonging to serotype H5a, 5b. We concluded that CFA analysis is a useful technique to determine if future human isolates identified as *B. sphaericus* in fact belong to other species of bacteria or whether the isolates originated from commercial products.

Bacillus sphaericus (Meyer and Neide) is a heterogeneous species based on DNA homology (14) containing strains that have commercial potential as mosquito larvicides. In 1980, *B. sphaericus* was divided into six groups (I, IIA, IIB, III, IV, and V) based on DNA homology; all larvicidal strains belong to DNA homology group IIA. This entomopathogenic group is also distinct from the other DNA homology groups based on numerical analysis of auxanograms, rRNA gene restriction patterns, random amplified polymorphic DNA fingerprinting, and cellular fatty acid (CFA) analysis (1, 5, 9, 14, 17, 18, 23, 28). DNA homology group IIA can be divided into subgroups by serotyping and phage typing (5). Larvicidal activity of the most toxic strains is due to the production of two proteins of 41.9 and 52.4 kDa which act as a binary toxin. Many of the most toxic strains of *B. sphaericus* are members of serotype H5a, 5b, and one strain belonging to this serotype, strain 2362 (isolated from blackflies in Nigeria), is fermented commercially. The sole *B. sphaericus* larvicide registered in the United States is Vectolex (strain 2362; Abbott Laboratories, North Chicago, Ill.).

Concerns about the human safety of larvicides containing *B. sphaericus* have been raised by Dixon (6) and Drobniewski (7), based on several reports of fatal human infections (2, 3, 7, 10). However, the role played by *B. sphaericus* in these fatalities is questionable. First, in all cases, the DNA homology group(s) that these human isolates belonged to is unknown. Second, with the exception of NCTC 11025, recovered post mortem from a pseudotumor of a lung (10), these isolates were not deposited in any collection; consequently, their mammalian pathogenicity cannot be confirmed. The importance of this last point is underscored in a case reported by Allen and Wilkinson (2). Although bacilli identified as *B. sphaericus* were isolated from antemortem cultures of blood, spinal fluid, and urine, these isolates were not pathogenic when inoculated intravenously and intraperitoneally into young rabbits.

In contrast to the case reports mentioned above, the pathogenicity of several isolates belonging to DNA homology group IIA (serotypes H2a, 2b; H5a, 5b; and H25) has been tested by the World Health Organization, as well as by industry. Rats, euthymic and athymic mice, and rabbits were exposed by oral administration; inhalation; ocular irritation; and intraocular, subcutaneous, intraperitoneal, and intracerebral injection. Some of these studies also included the lung isolate NCTC 11025. None of the isolates tested, including NCTC 11025, were pathogenic (4, 20, 21). Despite these safety studies, it seems likely that with increased use of microbial insecticides and concomitant human exposure to these products, further concerns will be raised.

The purpose of this study was to answer two questions. First, what is the relationship between NCTC 11025 and *B. sphaericus* 2362? Second, can we create a unique profile or "fingerprint" for *B. sphaericus* 2362, so that it can be distinguished from other strains within serotype H5a, 5b, as well as other DNA homology groups? Here we report the utility of CFA analysis for answering these questions.

MATERIALS AND METHODS

Sources of commercial and collection larvicidal cultures of serotype H5a, 5b. Vectolex CG (Abbott Laboratories), formulated on corncob granules, was received in 1990 and 1995 (see Table 1, strain 2362, lots 29-087-K1 and 08-640-N8). Samples were taken from these two time periods to confirm that there was no

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change in Vectolex due to either inadvertent or purposeful selection for clones that grow best under industrial fermentation conditions. *B. sphaericus* was isolated from this larvicide by shaking the granules onto brain heart infusion (BHI) agar plates (Micro Diagnostics, Addison, Ill.) and then incubating the plates at 28°C for 24 h. The following cultures were obtained from the collection of the Pasteur Institute (Paris, France): SPH 88 (current International Potency Standard for strain 2362, lot BSP 247A), SOS 010 (reference strain for strain 2362), SOS 214 (isolated from a European larvicide, Spherix, strain 2362), SOS 001 (reference strain for serotype H5a, 5b, strain 1593), and SOS 002 (strain 1593-4). SPH 88 was received as a powder, and this powder was shaken onto BHI agar plates, which were incubated as described above. The other cultures from the Pasteur Institute were received as spore-impregnated filter strips. The filter strips were placed onto BHI agar plates and rehydrated with BHI broth (Micro Diagnostics). The plates were incubated as described above.

Sources of nonlarvicidal *B. sphaericus* cultures. The type strain for *B. sphaericus*, ATCC 14577, was received as a lyophilized pellet from the American Type Culture Collection (Rockville, Md.). The culture was revived by suspending the pellet in BHI broth at 28°C for 24 h, and then 0.1 ml was streaked onto BHI agar plates, which were incubated at 28°C for 24 h. The following DNA homology group reference strains (agar slants) were received from A. A. Yousten, Virginia Polytechnic Institute and State University, Blacksburg: ATCC 7055 (reference strain for DNA homology group IIB), NRS 592 (reference strain for DNA homology group III), NRS 400 (reference strain for DNA homology group IV), and NRS 1198 (reference strain for DNA homology group V). A loopful of material from each slant was transferred to BHI agar plates and incubated as described above.

Source of human isolate. Two cultures of NCTC 11025 (isolated from a human lung) were received as lyophilized pellets from the National Collection of Type Cultures (Central Public Health Laboratory, London, United Kingdom). The cultures were revived by suspending each lyophilized pellet in BHI broth and incubating the suspension at 25°C for 4 days. After this incubation period, 0.1 ml of the BHI broth was streaked either onto tryptic soy broth agar (TSA) plates or TSA plates supplemented with 5% sheep blood (TSAB plates; Remel, Lenexa, Kans.) that were then incubated at 28°C. NCTC 11025 was not streaked onto BHI agar plates because of the prolonged incubation in BHI broth.

Other *Bacillus* species used in this study. The type strain of *B. globisporus*, ATCC 23301, was received from the American Type Culture Collection as a lyophilized pellet. The culture was revived by suspending the pellet in BHI broth at 28°C for 24 h, and then 0.1 ml was streaked onto TSAB plates, which were incubated at 28°C.

CFA sample preparation and analysis of data. Single-colony isolates of *B. sphaericus* NCTC 11025 and *B. globisporus* were obtained by using a four-quadrant streak pattern and then transferring single colonies to either TSA or TSAB plates. These plates were incubated at 28°C for 24 or 48 h in accordance with the standard TSA protocol of MIDI Corporation (Newark, Del.). Initially, all of the *B. sphaericus* samples were incubated for 24 h on TSA plates, and colonies of NCTC 11025 derived from a single lyophilized pellet were incubated for 48 h because of slow growth. In a second experiment, NCTC 11025, derived from a second lyophilized pellet, and *B. globisporus* were incubated on TSAB plates for 48 h. Colonies obtained from this second pellet of NCTC 11025 were also incubated on TSA plates for 48 h to compare their fatty acid values to the profiles created from the first pellet.

Approximately 40 to 50 mg (wet weight) of cells in the early stationary phase was then harvested from each plate, and the fatty acids were extracted and methylated (9, 16, 19, 24). The fatty acid methyl esters were identified with a Hewlett-Packard (Avondale, Pa.) Microbial Identification System HP 5898A, consisting of a 5890A gas-liquid chromatograph equipped with a 5% phenylmethyl silicone capillary column, a flame ionization detector, a 7673 automatic sampler, a 7673A controller, a 3392A integrator, and a Hewlett-Packard 300 computer. The gas-liquid chromatograph was calibrated every 11th vial with a Hewlett-Packard calibration standard kit containing fatty acid methyl esters in 0.8 ml of hexane (saturated nC9:0 to nC20:0 plus 2 and 3 hydroxy). A reagent control was included with every run. The species determination for each plate was based on a software library (TSBA version 3.8) developed by MIDI Corporation that compared the fatty acid composition of the extracted plates to library values. A separate computer record was generated for each plate, and we refer to these records as entries (9, 22, 24, 27).

Profile creation. The phenetic relationship among isolated colonies of each strain was determined by using Euclidean distance. Euclidean distance is a multivariate measure of the distance between variables and is calculated by using the Pythagorean theorem (15). We did not standardize the values used in our calculations because they were all in the same scale, percentage. Dendrograms were generated by using the library generation software (version 1) developed by MIDI Corporation, as well as the cluster analysis platform in JMP (SAS Institute Inc., Cary, N.C.). The hierarchical clustering method employed was the unpaired-group method using arithmetic averages (UPGMA). We began our analysis by clustering entries from a single accession number or single lot of *B. sphaericus*. Entries that linked at a Euclidean distance of ≤2.0 U were considered to belong to the same strain, based on empirical data from MIDI Corporation, as well as our own experience (22, 24). Subsequently, these individual entries were grouped into a new file that we refer to as a profile. Each profile contains the average value for every CFA identified in at least two plates. The next stage of our analysis examined the relationship among profiles by using UPGMA analysis. Profiles that linked at a Euclidean distance of ≤2.0 U were considered to belong to the same strain, and profiles that linked at a Euclidean distance of >10.0 U were considered to belong to different species, based on empirical data collected by MIDI Corporation (22, 24). We then validated our profiles by examining histograms of the primary and second and third most common CFAs obtained from the individual colonies that comprised our profiles, as well as the coefficient of variation (CV) of the principal fatty acid. The CV is the quotient of the standard deviation divided by the mean. Profiles were considered valid if the CV of the principal fatty acid was ≤2% (22, 24).

Reproducibility. The reproducibility of our method was tested by analyzing the data for 200 separate injections of the calibrator mixture with the cluster option in JMP and determining the Euclidean distance at which all of the calibrator entries linked together. We also evaluated the reproducibility of this method by comparing 10 plates of NCTC 11025 obtained from the second lyophilized pellet (incubated at 28°C for 48 h on TSA) to our profiles for NCTC 11025 that were created from the first lyophilized pellet.

Comparison of profiles for NCTC 11025 and existing literature values. The CFA values for our NCTC 11025 profiles were compared to values reported by Kämpfer (11) for the genus *Bacillus*. A set of data containing both our values and those of Kämpfer was created. The phenetic relationships within this set of data between NCTC 11025 and other species of bacilli were calculated by using the cluster analysis program in JMP. The type strain of the species in this database closest to NCTC 11025 was then obtained from the American Type Culture Collection. Single colonies of this type strain were isolated and incubated, and their CFAs were extracted as described above. Entries for the colonies were grouped into profiles as described above. The Euclidean distance between NCTC 11025 and this new *Bacillus* species was calculated to determine if NCTC 11025 belongs to this other species.

Numbers of plates analyzed. Two hundred sixty-seven plates were used in this study to create profiles as follows: 59 plates of NCTC 11025, 15 plates of SOS 010, 13 plates of Vectolex 1995, 16 plates of Vectolex 1990, 19 plates of SOS 214, 29 plates of SPH 88, 9 plates of SOS 001, 14 plates of SOS 002, 15 plates of ATCC 14577, 15 plates of NRS 592, 16 plates of NRS 400, 14 plates of ATCC 7055, 16 plates of NRS 1198, and 29 plates of *B. globisporus*. Multiple samples were run on several dates to maximize heterogeneity.

Growth of NCTC 11025 for morphological description. NCTC 11025 was streaked on TSAB plates and incubated aerobically at 25°C for 9 days. Slides were made, Gram stained, and then examined with a compound microscope.

RESULTS

Descriptive statistics. NCTC 11025 was gram positive and had primarily straight rods, with occasional serpentine forms. Spores were spherical and terminal with bulging of the sporangia. Colonies were soft, up to 7 mm in diameter.

The identities of the fatty acids present in the profiles of *B. sphaericus* and NCTC 11025 incubated on TSA plates and their mean percent composition and CV are reported in Table 1. Fifteen fatty acids were used in this analysis. Four fatty acids (i15:0, a15:0, 16:1 ω7 alcohol, and i16:0) accounted for approximately 80% of the total fatty acid composition of the cytoplasmic membrane. One fatty acid (i15:1 at 5) appeared at trace levels, ≤0.32%, or was lacking in the plates analyzed. Another fatty acid could not be resolved and is either an i17:1 I or an a17:1 B fatty acid; this fatty acid was identified as summed feature 5 in our printout. The only isolate that could not be identified by the MIDI Corporation TSA database was NCTC 11025.

NCTC 11025 differed from all of the other *B. sphaericus* strains examined that were grown on TSA in both the identity of its primary fatty acid (a15:0 rather than i15:0) and its lack of two secondary fatty acids common to all of the other strains, i17:1 ω10c and i17:0. The percent composition of the 16:1 ω7 alcohol fatty acid in NCTC 11025 was also noticeably lower.

When NCTC 11025 was incubated on TSAB, an additional three fatty acids were present in the cytoplasmic membrane (Table 2). These fatty acids were 14:0, i17:1 ω5c, and 18:0 and together comprised 2.73% of the fatty acids identified. The fatty acid identified as summed feature 5 was missing.

Profile creation. NCTC 11025 and ATCC 14577 were polytypic and contained two strains each based on our analysis. The profile for the predominant strain in each isolate was designated A and the profile for the secondary strain was designated

TABLE 1. Mean percent composition and CV of CFAs for the profiles of *B. sphaericus* and NCTC 11025 incubated at 28°C on TSA plates

Strain	Mean % composition (CV) of:														
	i14:0	14:0	i15:1 ω1 5	i15:0	a15:0	15:0	i16:0 3OH	16:1 ω7 alcohol	i16:0	16:1 ω11c	16:0	i17:1 ω10c	i17:0	a17:0	SF 5[a]
NCTC 11025-A	8.32 (7.0)			5.13 (7.0)	66.74 (1.0)	0.81 (14.0)		3.55 (10.0)	4.66 (6.0)	1.11 (9.0)	0.97 (17.0)			6.36 (8.0)	2.01 (35.0)
NCTC 11025-B	7.81 (3.0)			3.76 (5.0)	68.49 (1.0)	0.62 (16.0)		2.97 (5.0)	5.08 (3.0)	1.08 (13.0)	1.01 (13.0)			6.75 (3.0)	1.79 (7.0)
SOS 010	2.34 (4.0)	0.54 (12.0)		66.40 (1.0)	4.17 (7.0)	0.51 (71.0)		9.50 (1.0)	5.05 (3.0)	2.37 (7.0)	0.65 (34.0)	2.34 (3.0)	4.09 (4.0)	0.77 (10.0)	0.69 (14.0)
SPH 88	2.36 (5.0)	0.55 (6.0)		66.93 (1.0)	4.46 (5.0)	0.42 (57.0)	0.50 (9.0)	9.01 (5.0)	4.96 (5.0)	2.35 (5.0)	0.54 (22.0)	2.11 (7.0)	3.86 (5.0)	0.77 (9.0)	0.58 (21.0)
Vectolex 1990	2.30 (7.0)	0.56 (12.0)		65.62 (1.0)	4.41 (6.0)	0.49 (67.0)	0.35 (60.0)	9.48 (3.0)	5.39 (7.0)	2.47 (5.0)	0.62 (20.0)	2.35 (6.0)	4.05 (5.0)	0.84 (33.0)	0.75 (11.0)
Vectolex 1995	2.16 (4 0)	0.42 (3.0)		66.52 (1.0)	4.07 (4.0)	0.74 (10.0)	0.50 (33.0)	9.34 (2.0)	5.18 (5.0)	2.18 (4.0)	0.52 (10.0)	2.38 (4.0)	4.39 (4.0)	0.77 (7.0)	0.64 (25.0)
SOS 214	2.22 (7.0)	0.51 (7.0)	0.32 (5.0)	66.50 (1.0)	4.07 (4.0)	0.66 (17.0)	0.48 (4.0)	9.02 (3.0)	4.90 (4.0)	2.47 (5.0)	0.49 (10.0)	2.61 (6.0)	3.89 (5.0)	0.68 (11.0)	0.64 (25.0)
SOS 001	2.26 (5.0)	0.56 (4.0)	0.28 (15.0)	66.96 (1.0)	5.58 (6.0)		0.73 (6.0)	8.26 (4.0)	4.20 (2.0)	2.46 (4.0)	0.52 (35.0)	2.19 (7.0)	3.36 (8.0)	0.96 (11.0)	0.72 (38.0)
SOS 002	1.91 (4.0)	0.68 (4.0)		63.75 (2.0)	8.07 (5.0)		0.51 (8.0)	8.25 (12.0)	4.14 (5.0)	3.08 (4.0)	0.54 (14.0)	1.91 (4.0)	4.09 (7.0)	1.56 (12.0)	1.02 (5.0)
ATCC 14577-A	3.44 (6.0)			51.03 (1.0)	3.27 (4.0)			15.84 (5.0)	11.51 (5.0)	2.82 (6.0)	0.55 (35.0)	4.64 (7.0)	4.88 (4.0)	0.62 (31.0)	1.02 (6.0)
ATCC 14577-B	4.11 (6.0)	0.32 (4.0)	0.31 (0)	48.13 (1.0)	3.04 (6.0)			17.43 (4.0)	13.59 (3.0)	2.58 (3.0)	0.46 (1.0)	4.00 (4.0)	4.58 (2.0)	0.56 (6.0)	0.89 (9.0)
ATCC 7055	2.51 (5.0)	0.51 (7.0)		51.67 (1.0)	10.14 (4.0)	0.72 (16.0)	0.33 (10.0)	11.16 (2.0)	9.07 (2.0)	2.77 (5.0)	0.72 (5.0)	1.36 (4.0)	4.64 (3.0)	2.70 (7.0)	1.26 (4.0)
NRS 592	5.62 (2.0)	0.60 (4.0)		54.78 (0)	7.81 (5.0)	1.00 (9.0)		12.65 (1.0)	8.92 (3.0)	1.98 (2.0)	0.44 (6.0)	1.03 (3.0)	2.31 (3.0)	1.16 (6.0)	1.00 (4.0)
NRS 400	5.32 (3.0)	0.45 (5.0)		51.63 (1.0)	8.35 (2.0)	0.85 (13.0)	0.49 (4.0)	12.79 (1.0)	11.57 (3.0)	1.82 (4.0)	0.68 (8.0)	0.91 (3.0)	2.67 (3.0)	1.52 (4.0)	0.72 (3.0)
NRS 1198	2.03 (9.0)	0.31 (9.0)		38.72 (2.0)	6.28 (7.0)	0.82 (6.0)		20.36 (4.0)	17.14 (2.0)	2.73 (9.0)	0.61 (14.0)	1.79 (4.0)	4.56 (9.0)	2.18 (16.0)	2.27 (9.0)

[a] Summed feature (SF) 5 is either 17:1 iso I or 17:1 anteiso B.

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TABLE 2. Mean percent composition and CV of CFAs for the profiles of NCTC 11025 and *B. globisporus* incubated at 28°C for 48 h on TSAB plates

Strain	Mean % composition (CV) of:																
	i14:0	14:0	Unk[a] 14.258	i15:0	a15:0	15:0	16:1 ω7 alcohol	16:0 N alcohol	i16:0	16:1 ω11c	16:0	i17:1 ω5c	i17:0	a17:0	18:0	19:0 methyl	SF 5[b]
NCTC 11025	5.64 (4.0)	0.35 (41.0)		4.79 (8.0)	67.22 (1.0)	0.54 (28.0)	2.42 (6.0)		3.57 (5.0)	2.54 (9.0)	2.72 (7.0)	1.12 (81.0)		6.63 (4.0)	1.26 (12.0)		
ATCC 23301-A	3.00 (5.0)	0.71 (12.0)	0.39 (66.0)	4.65 (4.0)	53.90 (1.0)	0.56 (12.0)	2.32 (9.0)	0.46 (48.0)	2.56 (9.0)	6.42 (8.0)	5.92 (11.0)	3.90 (10.0)	0.77 (9.0)	9.46 (6.0)	3.9 (16.0)	0.55 (35.0)	
ATCC 23301-B	3.04 (2.0)	0.98 (0.0)		4.85 (7.0)	53.18 (2.0)	0.60 (4.0)	1.90 (3.0)	1.90 (3.0)	2.54 (4.0)	7.03 (4.0)	7.16 (3.0)		0.84 (2.0)	9.04 (1.0)	4.28 (9.0)		3.15 (9.0)
ATCC 23301-C	3.21 (10.0)	0.97 (12.0)		4.13 (6.0)	51.89 (1.0)	0.61 (13.0)	1.58 (2.0)	0.63 (3.0)	2.21 (5.0)	7.30 (1.0)	8.36 (5.0)	2.57 (7.0)	0.85 (9.0)	8.11 (1.0)	6.28 (12.0)	0.69 (17.0)	
ATCC 23301-D	2.81 (3.0)	0.93 (10.0)		4.37 (7.0)	50.97 (0.0)	0.72 (32.0)	1.65 (2.0)		2.35 (1.0)	6.48 (17.0)	7.57 (5.0)	2.74 (10.0)	0.79 (0.65)	8.99 (11.0)	4.53 (7.0)	0.78 (3.0)	
ATCC 23301-E	3.46 (5.0)	0.74 (24.0)		5.03 (11.0)	56.27 (0.0)		2.76 (1.0)		2.62 (11.0)	5.64 (8.0)	4.92 (13.0)	4.28 (9.0)	0.69 (17.0)	9.10 (2.0)	3.21 (0.0)	0.39 (0.0)	

[a] Unnamed fatty acid with equivalent chain length of 14.258.
[b] Summed feature (SF) 5 is either 17:1 iso I or 17:1 anteiso B.

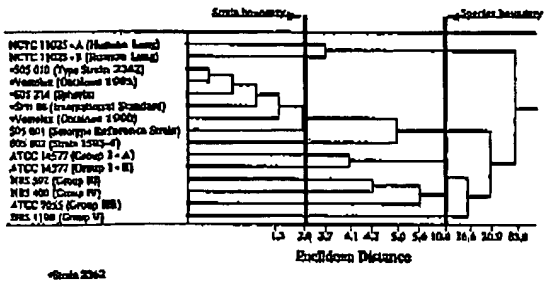

FIG. 1. UPGMA cluster dendrogram depicting the relationship between profiles of *B. sphaericus* derived from public culture collections, laboratory collections, and a commercially produced mosquito larvicide, Vectolex. This dendrogram was created with the cluster platform of JMP (SAS Institute).

B. NCTC 11025-A accounted for 72.7% (34 of 43) of the plates analyzed, and ATCC 14577-A accounted for 86.7% (13 of 15) of the plates analyzed. Both strains in NCTC 11025 formed a cluster at a Euclidean distance of 2.7 U, and both strains in ATCC 14577 formed a cluster at a Euclidean distance of 4.1 U.

A dendrogram depicting the relationship between all of our profiles derived from TSA is shown in Fig. 1. The profiles fell into two groups. The major group contained all isolates except NCTC 11025. These profiles linked together inside a Euclidean distance of 20.9 U. The minor group consisted of the two profiles of NCTC 11025. The NCTC 11025 profiles linked together at a Euclidean distance of 2.7 U and linked to the other profiles at a Euclidean distance of 83.8 U. These distances indicate that NCTC 11025 is not *B. sphaericus*.

Comparison between NCTC 11025 and *B. globisporus*. *B. globisporus* was polytypic when incubated on TSAB for 48 h. The profile for the predominant strain was designated A, and the profiles for the secondary strains were designated B, C, D, and E. *B. globisporus* A accounted for 72.4% (21 of 29) of the plates, and *B. globisporus* B through E accounted for 2 plates each. NCTC 11025 was monotypic under these conditions (16 plates) (Table 2). When the *B. globisporus* profiles were compared to that of NCTC 11025, the *B. globisporus* profiles linked together at a Euclidean distance of 5.9 U and linked to NCTC 11025 at a distance of 16.3 U. These distances indicate that although NCTC 11025 is not *B. globisporus*, it is more similar to *B. globisporus* than to *B. sphaericus*.

Reproducibility of the method. Of the 200 calibrator entries, 180 linked together inside a Euclidean distance of 0.1 U. All of the entries linked together inside a Euclidean distance of 0.5 U. The 10 plates obtained from the second lyophilized pellet of NCTC 11025 were identified by our NCTC 11025-A profile with similarity values of >0.8. These samples were then incorporated into the NCTC 11025-A profile.

DISCUSSION

Kaneda (12, 13) was the first to state that it is possible to create fingerprints of the genus *Bacillus* by using CFA analysis. The genus could be divided into major and minor groups based on the ratio of two fatty acids, i15:0 and a15:0. The a15:0 fatty acid, 12-methyltetradecanoic acid, is the predominant fatty acid in the major group, and the i15:0 fatty acid, 13-methyltetradecanoic acid, is the predominant fatty acid in the minor group. Other researchers have confirmed this finding (8, 9, 19, 22, 24, 25). More recently, Kämpfer (11) studied the fatty acid patterns of 313 *Bacillus* strains and Frachon et al. (9) examined 114 strains of *B. sphaericus* by using this technique. These researchers reported that in every strain analyzed, the i15:0 fatty acid predominated in *B. sphaericus*. The samples we analyzed (Table 1) are in agreement with these findings, except for NCTC 11025. The predominance of the a15:0 fatty acid in NCTC 11025 indicates that it was incorrectly identified as *B. sphaericus* by Isaacson et al. (10).

We evaluated the possibility that the predominance of the a15:0 fatty acid in NCTC 11025 was an artifact due to a longer incubation time by analyzing additional plates of *B. sphaericus* 2362 (obtained from Vectolex) incubated on TSA plates for 192 h at 28°C. There was no shift toward production of the a15:0 fatty acid; in fact, there was a 5% increase in the percentage of the i15:0 fatty. We conclude that the predominance of the a15:0 fatty acid in NCTC 11025 was not due to prolonged incubation. We also considered the possibility that the distant linkage of NCTC 11025 to the other profiles was dependent on the clustering algorithm used. We also calculated the Euclidean distance between profiles by using Centroid, Complete Linkage, Single Linkage, and Ward's clustering algorithms. In every instance, the NCTC 11025 profiles linked to the other profiles at Euclidean distances of >72 U.

When we incorporated the values of Kämpfer (11) into our set of data, *B. globisporus* linked to NCTC 11025 at a Euclidean distance of 10.3 U. Since this linkage occurred at the species boundary empirically derived by MIDI Corporation, we decided to examine the relationship between NCTC 11025 and *B. globisporus* by using our own profiles. This was necessary to standardize the growth medium and incubation time, both of which affect the identity and percentage composition of the fatty acids in the cytoplasmic membrane (13, 15, 21, 23). In addition, we were interested in determining if NCTC 11025 would still differ from *B. sphaericus* when grown on a medium commonly used in clinical diagnostic laboratories. NCTC 11025 was distinct from *B. globisporus* and remained unidentified; the a15:0 fatty acid still predominated.

We do not believe that our findings of heterogeneity in NCTC 11025, ATCC 14574, and ATCC 23301 indicate a problem with the reproducibility of CFA analysis. Our data for 200 separate injections of the calibrator (17 fatty acids) linked inside our empirically derived strain boundary of 2.0 Euclidean distance units. Our CV for the fatty acids in the calibrator ranged from 0.2 to 1.8. We conclude that the observed heterogeneity was not an artifact. Perhaps the samples selected for culture were mixed from the beginning and several clonal populations were then maintained in culture. Alternatively, the isolates selected for ATCC 14574, ATCC 23301, and NCTC 11025 may have initially been single colonies that subsequently mutated. It is difficult to reconcile our findings for these three samples with the standard definition of a strain, i.e., "the descendants of a single isolation in pure culture and usually made up of a succession of cultures ultimately derived from an initial single colony" (26). Our data suggest that caution is warranted when analyzing material obtained from culture collections, but these findings do not affect our conclusion that NCTC 11025 is not *B. sphaericus*.

There has been an evolution in the use of CFA analysis, as technological advances have enabled large sets of data to be analyzed. Kaneda (12, 13) concentrated on the i15:0/a15:0 fatty acid ratio in the genus *Bacillus* and ignored all of the other fatty acids. Esnard et al. (8) utilized all of the fatty acids identified but did not use the statistical technique of cluster analysis. Kämpfer (11) utilized all of the fatty acids identified and employed UPGMA clustering techniques but created profiles with unacceptable variation (as high as 49%) for the predominant fatty acid. Frachon et al. (9) and Schenkel et al. (18) used methods that approximate ours but did not specify

whether their fatty acid extracts came from single-colony isolates and also did not report the CV for the primary fatty acid. We believe that our approach to profile creation is unique and that the technique of CFA analysis has the greatest power of discrimination when a single colony is streaked per plate and the data are clustered by using hierarchical UPGMA analysis.

In summary, NCTC 11025 was misidentified as *B. sphaericus*. If the concerns raised by Dixon (6) and Drobniewski (7) are based on the case report of Isaacson et al. (10), then they are unwarranted. We note that NCTC 11025 underwent mammalian safety testing and was not pathogenic (20), yet neither author cited the relevant report. Assessment of the significance of the other case reports of *B. sphaericus* infections in humans is complicated by our inability to obtain these clinical isolates.

CFA analysis was a useful tool that successfully distinguished among *B. sphaericus* DNA homology groups, as well as strains within a serotype. If *B. sphaericus* is isolated from humans in the future, it is possible, by using CFA characterization, to compare the new isolate(s) to our existing profiles for strain 2362. We believe that this technique is also applicable to other species of bacteria that are used as insecticides or fungicides.

ACKNOWLEDGMENTS

We are grateful to I. Thiery of the Pasteur Institute, A. A. Yousten of Virginia Polytechnic Institute and State University, and B. Melin of Abbott Laboratories for providing cultures and larvicide. We thank M. H. Vodkin, J. V. Maddox, and four anonymous reviewers for reading the manuscript.

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ATCC® connection™

2003

Characterization of Bacteria by Ribotyping

Jane Tang, Ph.D., David Cleland, and David Emerson, Ph.D., ATCC

A major area of research in microbial characterization has been the development of molecular methods for genotyping organisms. Genotypic methods can be highly specific and sensitive and are largely independent of the physiological or growth state of the organism.

Ribotyping, a molecular method based on the analysis of restriction fragment length polymorphisms (RFLPs) of ribosomal RNA genes, has been widely adopted to distinguish a variety of bacteria (1-5). For the past five years ATCC has been using the RiboPrinter® Microbial Characterization system (Qualicon Inc., Wilmington, DE) as a primary method for characterizing strains during the quality control process.

The RiboPrinter is a molecular workstation that performs a restriction digest (using *EcoR*I or other restriction enzymes) of the chromosomal DNA, separates the restriction fragments by gel electrophoresis, and simultaneously blots the DNA fragments to a membrane which is used for Southern blot analysis. Restriction digest fragments are hybridized to a bacterial probe that is based on the conserved regions of the genes for the ribosomal DNA operon. The result is a DNA fingerprint which is strain specific. Each fingerprint is stored in a database so it can be accessed for future comparisons and identifications.

We routinely use the RiboPrinter system in several steps of our quality control and authentication process for bacterial cultures. Ribotyping is per-

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ATCC Antibiotic Mix for Establishing Primary Cultures and Cell Lines

Ariane Thompson, Yvonne Reid, Ph.D., and Deborah Polayes, Ph.D., ATCC

Primary cellular material, especially epidermis, often contains bacteria and fungi that must be removed to ensure successful establishment of a new cell line. ATCC has developed Antibiotic Mix (catalog no. 30-2306) for use in generating primary cell lines, and it has been used extensively at ATCC in the preparation of primary fibroblasts (Table 1).

Preparation of primary cells and subsequent cell line establishment at ATCC follow the procedures described in *Culture of Animal Cells*, 4th ed., by R.I. Freshney (New York: Wiley-Liss; 2000). Adding an antibiotic mix greatly decreases the incidence of microbial contamination and increases your chance of establishing pure cell cultures.

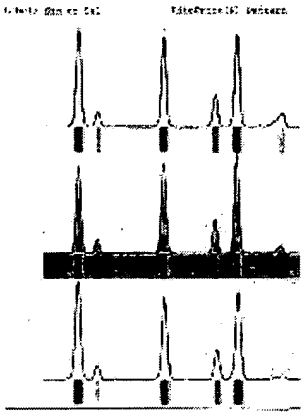
Protocols for establishing cell cultures (including the dissociation enzymes, volume and type of medium, centrifugation times, and amount of antibiotic mix) vary significantly depending on the type of cell and the quantity of starting material. ATCC's procedure for preparing primary human fibro-

continues on page 6 ▷▷

In This Issue

Ribotyping
◁◁continued from p. 1

Figure 1. Three riboprints of *Xanthomonas oryzae* BAA-145. The top print was from the original culture when it arrived at ATCC, the middle from a seed lot, and the bottom from a distribution lot, showing no change as the strain was processed.



formed when the culture arrives at ATCC and the results then become part of the laboratory record. DNA fingerprints from future seed stock and replenished materials are compared with this original to ensure that the culture has not changed during the propagation and preservation processes (Figure 1). Over the last few years we have tested hundreds of genera and species using this automated genotyping method and the results indicate that the system is reliable and reproducible.

Ribotyping is very useful for authenticating fastidious organisms, which are often difficult to characterize by traditional physiological methods. Ribotyping patterns are generated from both environmental strains and clinical isolates and the records augment our characterization profiles. Since we accession many newly discovered and published bacteria, we do not expect the system to produce identification because the new names are not yet in the existing database. Rather we examine and archive the DNA fingerprint patterns to verify authenticity.

Characterization and relatedness

Recently we used the RiboPrinter system to survey members of the Legionellaceae family (6). The majority of *Legionella* species are currently typed by traditional serological methods. Our study, which included 110 strains comprising 48 genera and 70 serogroups, showed that the ribotyping system works well in distinguishing members of this genus. Strains within the same species displayed consistent patterns even when isolated from a variety of sources and geographic locations, yet species were clearly differentiated. We also provided examples where this system could be used to identify new isolates by comparing their patterns to those generated from known *Legionella* species.

In another research application we used the Riboprinter to genotype a group of isolates from salt marsh sediments that belonged to the classes *Flavobacteria* and *Sphingobacteria* in the phylum *Bacteroidetes*. These bacteria are now being recognized as some of the most common members of both marine and soil habitats, yet little is known about their population structure. Riboprint analysis showed that these strains exhibited significant genotypic diversity despite a more limited phenotypic diversity (7).

Studying morphological variants

Ribotyping is also very useful for resolving problems of colony variation within a strain. For example, members of the genus *Bacillus* are infamous for producing a variety of colonies types (i.e., wrinkled vs. smooth). Ribotyping can confirm that the differing colonies originated from the same organism and are not contaminants (Figure 2).

Another example of the utility of this system is demonstrated in the analysis of a recent ATCC accession, *Rhodospirillum indiensis* BAA-36. This organism undergoes dramatic changes in cell morphology during aerobic growth, from spiral-shaped rods to curved short rods to packets of cocci, giving the appearance that the culture was contaminated (8). However, a repeated growth experiment coupled to analysis of the different growth stages by riboprinting proved that the different cell shapes were indeed the same organism (Figure 3).

The RiboPrinter system allows the flexibility in using other restriction enzymes besides the standard *EcoR*I. Some genera yield better patterns with *Pvu*II or *Pst*I. Other restriction enzymes can also be substituted in the process.

Careful, thorough characterization is a mainstay of ATCC's collection of prokaryotes, and ribotyping is a method that works with a broad range of organisms. Other advantages include the ease of operation and data analysis. The system requires only a single colony as inoculum and there are no restrictions on media and growth conditions. The adaptability and flexibility of the system make it a valuable part of ATCC's overall quality control program.

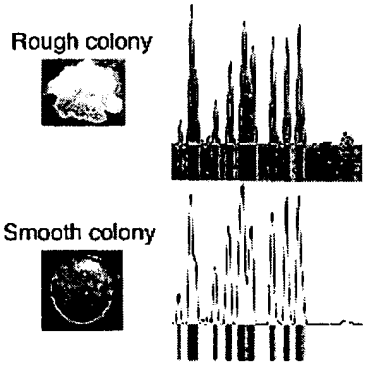


Figure 2: Riboprints of two colony variants of *Bacillus subtilis* 6051.

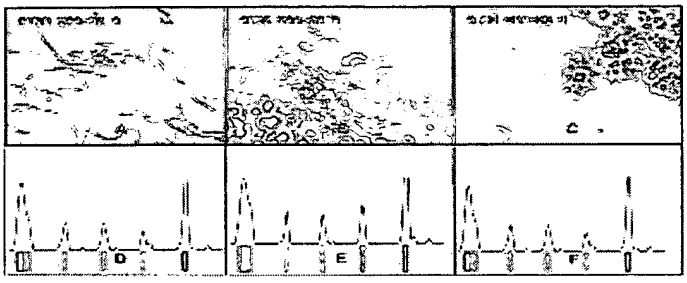


Figure 3. Ribotyping patterns of different cell types occurring during aerobic growth of *Rhodospirillum indiensis* BAA-36. Image A was taken after 1 to 2 days of growth, image B after 3 to 4 days, and image C after 6 to 7 days. The accompanying riboprints show that the different morphologies are the same organism.

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Authentication 101: Testing of Prokaryotes at ATCC

In this issue of *ATCC Connection* we describe just one of many procedures we use to characterize microorganisms. Given the great diversity of prokaryotes at ATCC it is a constant challenge to streamline the authentication process so that microbes can be characterized with the greatest efficiency. By utilizing several diverse identification strategies at the phenotypic and genotypic levels we have developed protocols that ensure thorough characterization of every strain.

The first step is to check the growth, purity, and cell and colony morphologies of all cultures that arrive at ATCC for deposit. Cultures then undergo biochemical testing if appropriate. For many of the more common bacteria we have developed a standard set of growth and biochemical tests that are based on well-known traits of these organisms. These biochemical tests, which include the use of API strips (bioMérieux, Inc.) and other commercial rapid tests, have been developed for different bacterial groups. We have refined these schema over the years to minimize the number of tests yet still provide robust identification. Even with these refinements there are 23 different characterization schemes encompassing nearly 350 individual tests that are performed on a routine basis.

The biochemical tests are instrumental for authenticating many important phenotypic properties of ATCC microbes. They are time-consuming, however, and the repertoire of biochemical tests is very limited for some organisms. Therefore, whenever possible we take a polyphasic approach to authentication that elucidates both phenotypic and genotypic traits of the organism. We are constantly evaluating and utilizing new technologies that balance selectivity, throughput, cost, and effort to ensure the best quality for our authentication procedures.

Over the next several issues we will describe other automated and molecular-based methods used at ATCC to characterize bacteria. To see an overview of the whole QC process, go to the bacteria search page on our Web site and look for the link under the query window.

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Identification of the *Staphylococcus sciuri* Species Group with *Eco*RI Fragments Containing rRNA Sequences and Description of *Staphylococcus vitulus* sp. nov.

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Strains of a new species, *Staphylococcus vitulus*, were isolated from food and a variety of mammals. This species was recognized on the basis of the results of an analysis of genomic *Eco*RI fragments containing portions of the rRNA operons. The patterns of hybridized fragments obtained from strains belonging to the new taxon were sorted into a distinguishable cluster and were distinct from the *Staphylococcus lentus* and *Staphylococcus sciuri* patterns. The results of DNA-DNA hybridization reactions demonstrated that strains in this cluster were more closely related to *S. lentus* and *S. sciuri* than to other *Staphylococcus* species and yet were significantly different. While these strains had some of the phenotypic characteristics of the *S. sciuri* species group, the newly recognized taxon could be distinguished by its very small colonies on P agar, absence of alkaline phosphatase activity, and lack of acid production from L-arabinose, maltose, *N*-acetylglucosamine, D-mannose, and raffinose. The type strain of the new species is strain DD 756 (= ATCC 51145).

A general method for distinguishing bacterial species by using restriction fragments containing portions of their rRNA operons has been described previously (12, 26, 31). This method has been applied to the genus *Staphylococcus* (8, 9, 29) and recently was recommended as a way to distinguish a newly described staphylococcus from previously described taxa (7).

In this study, the electrophoretic patterns of restriction fragments labeled by hybridization with an rRNA operon from *Escherichia coli* were used to characterize organisms belonging to the *Staphylococcus sciuri* species group. When the patterns were sorted on the basis of similarity by using correlation values, clusters of strains identified as *S. sciuri* and *Staphylococcus lentus* were formed. We also distinguished another cluster of novobiocin-resistant, oxidase-positive staphylococci. This third taxon and its relationship to the *S. sciuri* species group are described in this paper.

MATERIALS AND METHODS

Bacterial strains. In this study, strains were identified by their DuPont numbers. Table 1 shows the strains which we studied, other designations of some strains, the species or subspecies to which each strain belongs, and the source of each strain.

Characteristic determinations. The following characteristics were determined as previously described (18, 19, 21, 22): colony morphology and pigment, motility, anaerobic growth in thioglycolate broth, catalase activity, acetylmethylcarbinol (acetoin) production, nitrate reduction, tube coagulase activity, clumping factor, hemolysis of bovine blood, carbohydrate reactions, and susceptibility to various antibiotics. Clumping factor and protein A were detected with a Staph Latex kit (Remel, Lenexa, Kans.). The oxidase test was performed by using a Microdase disk (Remel) (10). Pyrrolidonyl arylamidase activity was determined by using the Pyr broth and Pyr reagent of Carr-Scarborough Microbiologicals (Stone Mountain, Ga.) for identification of group A streptococci and enterococci (13). Esculin hydrolysis was determined on Aesculin agar (Carr-Scarborough Microbiologicals). Heat-stable nuclease activity was analyzed by using thermonuclease agar supplemented with toluidine blue (Remel) according to the manufacturer's instructions. Ornithine decarboxylase activity was determined by using a modification of the test of Moeller (25), as described in the *Manual of Clinical Microbiology* (18). Alkaline phosphatase, urease, β-galactosidase, β-glucosidase, and β-glucuronidase activities and arginine utilization were analyzed by using the API STAPH-IDENT system (bio-Merieux Vitek, Hazelwood, Mo.). Additional biochemical profile data were obtained by using the STAPH Trac system (bio-Merieux Vitek).

DNA-DNA hybridization. DNA was isolated and purified by using the procedures of Brenner and coworkers (5), as modified by Kloos and Wolfshohl (23). DNA-DNA hybridization reactions were performed under stringent (70°C) and optimal (55°C) conditions, and single- and double-stranded DNAs were separated by using the batch procedure for determining the extent of hybridization and thermal elution of DNA from hydroxyapatite (5).

Patterns: cell and DNA processing. Cells from 3 ml of broth were isolated and lysed in 10 mM Tris-HCl–10 mM sodium chloride–50 mM EDTA (pH 8.0) by sequentially adding 30 μg of *N*-acetylmuramidase, 600 μg of lysozyme, 25 U of lysostaphin, and 10 U of RNase (total volume, 70 μl). Following incubation, 800 μg of achromopeptidase in 40 μl of water was added. After a second incubation, 126 μl of a 10% sodium dodecyl sulfate (SDS) solution and 1.26 mg of proteinase K (concentration, 10 mg/ml) were added. The genomic DNA was isolated by phenol-chloroform-water extraction and ethanol precipitation. The DNA was digested to completion with *Eco*RI.

Patterns: electrophoresis, transfer, denaturation, and UV cross-linking. The DNA fragments were separated by electrophoresis in a 0.8% agarose gel in a minigel apparatus. The

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TABLE 1. *Staphylococcus* strains used, species designations, and sources of strains

Strain[a]			Bacterial species or subspecies	Source
DuPont no.	Kloos no.	Other designation		
DD 756[T]		ATCC 51145[T,b]	*S. vitulus*	Ground lamb
DD 763		ATCC 51162[b]	*S. vitulus*	Processed chicken
DD 768			*S. vitulus*	Beef, ground chuck
DD 771		ATCC 51163[b]	*S. vitulus*	Beef, ground chuck
DD 791			*S. sciuri*	Veal leg, sliced
DD 852[T]	GH137[T]	ATCC 29974[T]	*S. colnii* subsp. *colnii*	Human
DD 853[T]		ATCC 43958[T]	*S. equorum*	Horse
DD 854[T]		ATCC 35539[T]	*S. gallinarum*	Poultry
DD 857[T]	SC210[T]	ATCC 43959[T]	*S. kloosii*	Eastern gray squirrel
DD 863[T]		ATCC 12600[T]	*S. aureus*	Human
DD 864[T]		ATCC 14990[T]	*S. epidermidis*	Human
DD 866[T]		ATCC 15305[T]	*S. saprophyticus*	Human
DD 869[T]	KL162[T]	ATCC 29971[T]	*S. xylosus*	Human
DD 871[T]		ATCC 43957[T]	*S. arlettae*	Poultry
DD 1078		CFDRA MDW58	*S. vitulus*	Minced beef
DD 4201	OA542		*S. lentus*	Domestic sheep
DD 4202	K-15		*S. lentus*	Dairy goat
DD 4203[T]	K-21[T]	ATCC 29070[T]	*S. lentus*	Dairy goat
DD 4204	K-6		*S. lentus*	Dairy goat
DD 4277[T]	SC 116[T]	ATCC 29062[T]	*S. sciuri*	Eastern gray squirrel
DD 4522	H4F28		*S. vitulus*	Yearling horse
DD 4523	V51	ATCC 51161[b]	*S. vitulus*	Pine vole
DD 4524	VE 2		*S. vitulus*	Raw veal trimmings
DD 4525	VE 15		*S. vitulus*	Raw veal trimmings
DD 4535	RM112		*S. sciuri*	Black rat
DD 4751	BH2		*S. sciuri*	Beef head
DD 4756	BT12		*S. sciuri*	Beef trimmings
DD 4753	VE 23		*S. vitulus*	Raw veal trimmings
DD 5310	BC5F4		*S. lentus*	Beef cattle
DD 6049	TT4m1		*S. lentus*	Bottlenose dolphin
DD 6065	GMWg12		*S. vitulus*	Pilot whale

[a] ATCC, American Type Culture Collection, Rockville, Md.; CFDRA, Campden Food and Drink Research Association, Campden, United Kingdom.
[b] This strain was submitted to the American Type Culture Collection during preparation of the manuscript.

separated DNA fragments were transferred from the agarose gel to a nylon membrane (type NT4HY; Micron Separations, Westborough, Mass.). The DNA was then denatured, and the membrane was dried. The DNA was cross-linked to the nylon membrane by using UV light.

Patterns: probe preparation, hybridization, and detection. A plasmid containing the rRNA operon (*rrnB*) from *E. coli* (6) was linearized with *Eco*RI and labeled by using a sulfonation reaction (30).

The hybridization cocktail contained 125 μg of sonicated and denatured salmon sperm DNA per ml, 0.5 M sodium chloride, and 1% SDS. For each membrane, 1.5 μg of the sulfonated DNA was denatured and combined with 6 ml of hybridization cocktail. After overnight hybridization at 66°C, the membrane was washed with a 0.5 M sodium chloride–1% SDS solution at 66°C before it was dried.

The modified DNA probe was detected by using a conjugate consisting of anti-sulfonated DNA monoclonal antibody and alkaline phosphatase (15, 16). The monoclonal antibody (Orgenics, Yavne, Israel) was activated with *N*-succinimidyl-4-(*N*-maleimidemethyl)cyclohexane-1-carboxylate. Sulfhydryl groups were created on the alkaline phosphatase by using *N*-succinimidyl-5-acetylthiacetate and were deprotected with hydroxylamine. Conjugation of monoclonal antibody-maleimide with alkaline phosphatase containing sulfhydryl groups was accomplished by incubating the preparation in the dark. The conjugate was purified on a Zorbax GF-250 column.

After treatment with blocking buffer (30 g of skim milk powder [Difco Laboratories, Detroit, Mich.] per 100 ml of 25 mM NaCl–50 mM Tris-HCl [pH 7.5]–1 mM EDTA–0.3% Tween 20) and with the conjugate solution, the membrane was washed with assay buffer (50 mM sodium bicarbonate-carbonate, 1 mM magnesium chloride; pH 9.5). After the final wash fluid was decanted, 20 ml of assay buffer and 220 μl of chemiluminescent substrate solution PPD (10 mg/ml; Lumigen, Detroit, Mich.) were added to each membrane preparation. The membrane was removed from the solution, dried, and attached to a plastic frame, and chemiluminescent images were recorded electronically by using a high-sensitivity, supercooled Star One camera (Photometrics, Tucson, Ariz.) in a dark environment. The images were stored on a Macintosh IIci computer (Apple Computer, Cupertino, Calif.).

Patterns: data processing. For each membrane image, the software located the lane positions, reduced the background and noise, scaled each lane's image intensity, and used the data from the lanes containing DNA standards of known sizes to normalize the band positions. The normalized position and intensity profile for each lane, referred to as a pattern, was then stored as an individual record consisting of 512 bytes in a data base.

Additional custom software based on the method of Hubner (14) was used to analyze the levels of correlation between pairs of patterns. This software used the 512 intensity values for each lane as coordinates in a Euclidean, 512-dimensional space. Each pattern represented a single point in the 512-dimensional space. Each pair of patterns was compared by measuring the angle between the pair of lines constructed from the origin of the 512-dimensional space to each of the two points created

TABLE 2. Results of hybridization of staphylococcal DNAs with [*methyl*-^{3}H]thymidine-labeled DNAs

Species or subspecies	Pattern type[a]	Strain	% Relative binding with labeled DNA from:							
			DD 756^T		DD 771		DD 4525		DD 4523	
			55°C	70°C	55°C	70°C	55°C	70°C	55°C	70°C
S. vitulus	dd 756	DD 756^T	100	100	81	84	74	81	81	79
	dd 756	DD 4753	87	93	97	93	78	84	76	80
	dd 756	DD 6065	74	82	81	88	NT[b]	NT	NT	NT
	dd 771	DD 771	74	77	100	100	86	81	86	79
	dd 771	DD 1078	76	76	84	84	88	84	80	81
	dd 4522	DD 4522	75	75	88	81	88	83	85	84
	dd 4522	DD 4525	74	83	87	83	100	100	84	88
	dd 4523	DD 4523	79	76	85	81	81	82	100	100
S. sciuri		DD 4277^T	46	10	53	14	NT	NT	NT	NT
S. lentus		DD 4203^T	38	7	42	15	NT	NT	NT	NT
S. saprophyticus		DD 866^T	14	7	25	9	NT	NT	NT	NT
S. cohnii subsp. *cohnii*		DD-852^T	19	7	28	9	NT	NT	NT	NT
S. xylosus		DD 869^T	15	5	27	9	NT	NT	NT	NT
S. kloosii		DD 857^T	14	5	29	8	NT	NT	NT	NT
S. equorum		DD 853^T	16	6	25	9	NT	NT	NT	NT
S. urlettae		DD 871^T	17	8	28	10	NT	NT	NT	NT
S. gallinarum		DD 854^T	13	8	14	5	NT	NT	NT	NT

[a] Each pattern type was named after the strain with the lowest DuPont Company number that exhibited that pattern.
[b] NT, not tested.

from the two patterns being compared. Similar patterns had angles approaching zero degrees and cosines approaching unity (one). While the correlation was defined as the cosine of the angle, in this work we routinely used the correlation value squared.

Cell wall analysis. Staphylococci were grown to the stationary phase in a medium containing (per liter of distilled water) 10 g of tryptone, 5 g of yeast extract, 5 g of glucose, and 5 g of NaCl (pH 7.3). The cells were harvested by centrifugation at 20,000 × *g* for 20 min at 4°C and were disintegrated by shaking the preparation with glass beads. Cell walls were purified with 4% SDS by using the procedures of Glauner et al. (11) to determine peptidoglycan composition and teichoic acids. For total amino acid analysis cell walls purified with SDS were hydrolyzed with 4 N HCl for 16 h at 100°C. The levels of amino acids and amino sugars were determined by using a model LC6001 amino acid analyzer (Biotronik, Maintal, Germany). The peptidoglycan type was determined on the basis of the molar ratio of glutamic acid to L-lysine to L-serine to L-alanine to glycine (28). Teichoic acids were extracted from cell walls purified with SDS by the method of Kaya et al. (17). The teichoic acids were purified by ion-exchange chromatography on a column (1.6 by 17 cm) filled with DEAE-Sephacel (Pharmacia, Uppsala, Sweden), using a flow rate of 20 ml/h, a base mobile phase consisting of 0.01 M Tris-HCl (pH 7.0), and a linear 0 to 1 M NaCl gradient. The teichoic acids were hydrolyzed with 60% (wt/vol) hydrofluoric acid for 16 h at 0°C (3). Nonphosphorylated teichoic acid fragments were separated on a column (1.5 by 90 cm) filled with Bio-Gel P-2 (Pharmacia) by using a distilled water mobile phase and a flow rate of 8 ml/h. The sugars in the fractions were determined by gas-liquid chromatography, and glycerol was detected enzymatically by the method of Bergmeyer (4). The sugars were completely hydrolyzed with 2 N HCl for 3 h at 100°C (3). The hydrolysates were derivatized as described by Albersheim et al. (1) and were subjected to gas-liquid chromatography by using a Packard model GC 438 gas chromatograph equipped with a flame ionization detector (Packard Instrument Co., Meriden, Conn.) and a column (2 by 1,000 mm) filled with 3% SP 2340 on 100/200 Supelcoport (Supelco, Bellefonte, Pa.). The gas-liquid chromatography column was equilibrated at 140°C and was kept at that temperature for the first 2 min of each analysis. The temperature was increased at a rate of 6°C/min until it reached 270°C, where it was kept for an additional 3 min. Phosphate levels were determined by the method of Ames (2).

DNA base composition. The guanine-plus-cytosine content of DNA was determined by A. G. Steigerwalt in the laboratory of D. J. Brenner, Centers for Disease Control, Atlanta, Ga., by using the thermal denaturation method of Marmur and Doty (24).

RESULTS AND DISCUSSION

DNA-DNA hybridization. The DNA relationships among *Staphylococcus vitulus* strains that represented different pattern types and between *S. vitulus* and other *Staphylococcus* species are shown in Table 2.

In DNA-DNA hybridization reactions performed under optimal (55°C) and stringent (70°C) conditions, *S. vitulus* strains exhibited relatively high levels of DNA similarity (82% ± 6% at 55°C and 82% ± 4% at 70°C [means ± standard deviations]). No significant differences in levels of DNA similarity were found between *S. vitulus* strains that represented different pattern types. However, the levels of DNA relatedness between the new species and other members of the *S. sciuri* species group, including *S. lentus* and *S. sciuri*, were significantly lower. For *S. lentus*, the levels of DNA similarity were 40% ± 2% at 55°C and 11% ± 4% at 70°C. For *S. sciuri*, the levels of DNA similarity were 50% ± 4% at 55°C and 12% ± 2% at 70°C. The levels of DNA relatedness between *S. vitulus* and other novobiocin-resistant *Staphylococcus* species that did not belong to the *S. sciuri* species group were even lower.

Patterns. The raw image from a typical agarose gel generated by using enzyme-triggered chemiluminescence and captured with a customized charge-coupled device camera is shown in Fig. 1. The lanes that are not labeled with a species name were used to separate DNA standards whose sizes were known. The positions of the standard bands were used to

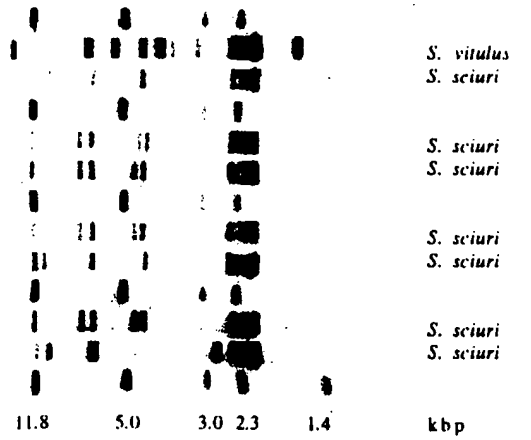

FIG. 1. Electronically recorded image of a membrane containing *Eco*RI fragments hybridized with a plasmid containing the *rrnB* rRNA operon of *E. coli*.

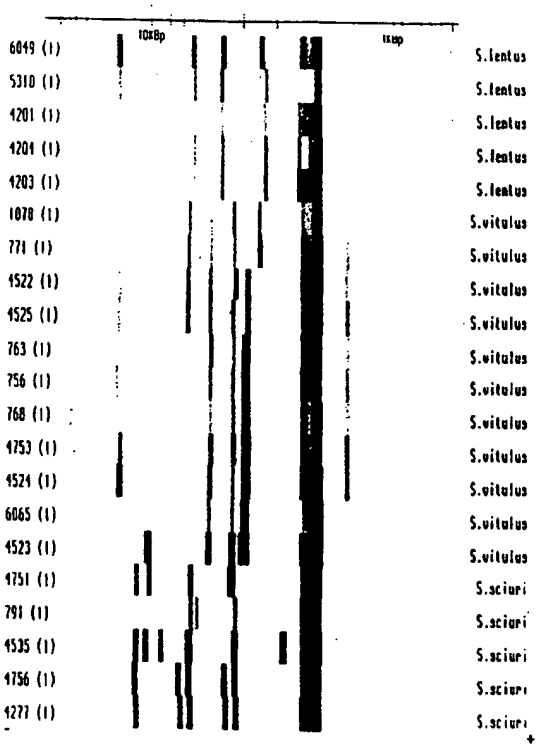

FIG. 2. Patterns of *Eco*RI fragments containing rRNA sequences derived from image data. The image data for each lane were processed to normalize band positions relative to standards, to reduce background, and to scale the band intensity. The pattern numbers are shown on the left, and the species names are shown on the right.

correct for both lane-to-lane and gel-to-gel variations in band position.

A processed image of some of the patterns determined during this study is shown in Fig. 2. The displayed width of each lane pattern was arbitrarily chosen for Fig. 2. The patterns were arranged to maximize the squared correlation values for adjacent patterns in the display. This sorting revealed that the *S. vitulus* patterns were most similar to the *S. lentus* and *S. sciuri* patterns and were different from the patterns obtained with the more than 200 other species in our data base. *S. vitulus*, *S. lentus*, and *S. sciuri* could be differentiated by their patterns either by visually comparing the patterns or by computer analysis.

Visually, the qualitative differences among the patterns of the three species were obvious. The *S. vitulus* patterns revealed sets of conserved, frequently occurring restriction fragments that were found in different strains of *S. vitulus* but were not conserved as a group in strains of other species.

Patterns that were identical (within the reproducibility of the method) were considered members of the same pattern type. Multiple strains belonging to the same species could have the same pattern type. Each pattern type was named after the lowest-numbered DuPont strain that produced that type of pattern. The 11 *S. vitulus* strains examined produced four distinguishable types of patterns, dd 756, dd 771, dd 4522, and dd 4523.

Computer analysis provided a way to measure the levels of similarity of the patterns by using the squared correlation values obtained from the 512-dimensional space. All of the *S. vitulus* patterns of the same pattern type (for example, dd 756) had squared correlation values with each other that were greater than 0.85. When each of the four *S. vitulus* pattern types with was correlated with its nearest *S. vitulus* neighbors in Fig. 2, the squared correlation values ranged from 0.71 to 0.79. No *S. vitulus* pattern had a squared correlation value with any *S. lentus* pattern that was greater than 0.56 or a squared correlation value with any *S. sciuri* pattern that was greater than 0.46.

Figures 3 and 4 show two-dimensional representations of the 512-dimensional space. The patterns of more than 5,500 strains, including members of approximately 200 species, were each defined by a single labeled point in Fig. 3 and 4. In each figure, the squared correlation values with the two reference patterns defined the two dimensions. For Fig. 3 we used the nomenclatural type strain of *S. lentus*, DD 4203, and the nomenclatural type strain of *S. vitulus*, DD 756, as the reference strains. For Fig. 4 we used the nomenclatural type strain of *S. vitulus*, DD 756, and the nomenclatural type strain of *S. sciuri*, DD 4277, as the reference strains. In these two-dimensional representations, the visual distinctions between the unique *S. vitulus* cluster and the other species tested are relatively clear. Clusters of points surrounding the reference strains are clearly identified by those reference strains.

Using the complete 512-dimensional-space analysis and the corresponding correlation values, we could clearly distinguish between *S. vitulus* and all of the other species tested. An organism could be identified as a member of *S. vitulus* by the squared correlation value of its pattern obtained from the 512-dimensional-space analysis, even if the pattern did not exactly match one of the pattern types associated with *S. vitulus*. In this study, squared pattern correlation values greater than 0.71 appeared to be strong evidence that strains belonged to the same species.

Cell wall peptidoglycan and teichoic acid. We also determined the peptidoglycan compositions of *S. vitulus* DD 756^T (T = type strain), DD 771, and DD 4523, *S. lentus* DD 4202, *S. sciuri* DD 4277^T, and *Staphylococcus aureus* DD 863^T. The peptidoglycan of *S. vitulus* strains is type L-Lys-L-Ala-Gly$_{3-3.4}$

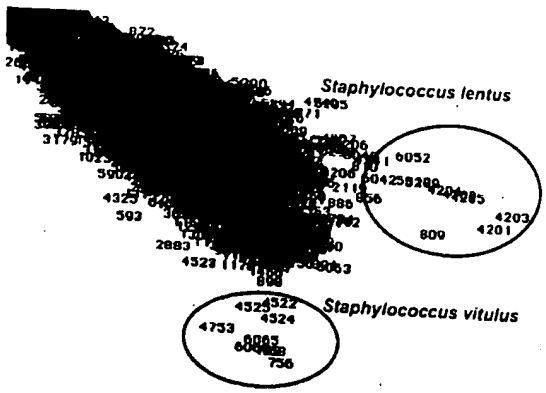

FIG. 3. Clustering of patterns as determined by transforming each pattern into a point in 512-dimensional space and depicting the points in a two-dimensional representation by using *S. lentus* DD 4203^T and *S. vitulus* DD 756^T to define the two dimensions. The strain number is placed in the appropriate location for each point on the figure. Data for approximately 5,550 strains belonging to 200 species are shown.

peptidoglycan. This peptidoglycan type is also present in *S. lentus* and *S. sciuri* (27).

The teichoic acids of *S. vitulus* are based on glycerol. Strain DD 756^T has a glycerol teichoic acid with an integrated *N*-acetylglucosamine 1-phosphate; strain DD 771 has a glycerol teichoic acid substituted at a high level with *N*-acetylgalactosamine; and strain DD 4523 has a glycerol teichoic acid substituted at a low level with *N*-acetylglucosamine.

DNA base composition. The guanine-plus-cytosine content of *S. vitulus* DD 756^T DNA is 34 mol%; the DNA guanine-plus-cytosine content of strains DD 771 and DD 4523 is 35 mol%; and the *S. sciuri* and *S. lentus* DNA guanine-plus-cytosine contents are between 30 and 36 mol% (20).

Description of *Staphylococcus vitulus* sp. nov. *Staphylococcus vitulus* (vit'u.lus. N. L. n. *vitulus*, veal). The description of *S. vitulus* below is based on the results of a study of 11 strains.

A total of 10 of the 11 strains produce colonies that are less than 3 mm in diameter when the organisms are grown aerobically on P agar (18) at 35°C. Colonies on P agar are usually raised with ulcerated, irregular centers, are opaque, and often have sectored or irregular edges; 10 of the 11 strains studied produce cream to yellow pigmentation, and 1 strain produces unpigmented colonies. Colonies grown on tryptic soy agar are much larger (8 to 12 mm in diameter) and not as irregular as colonies grown on P agar.

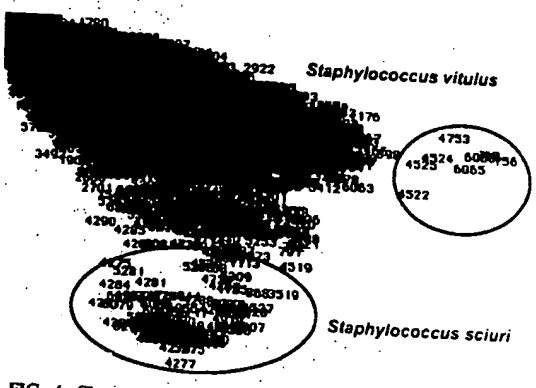

FIG. 4. Clustering of patterns as determined by transforming each pattern into a point in 512-dimensional space and depicting the points in a two-dimensional representation by using *S. vitulus* DD 756^T and *S. sciuri* DD 4277^T to define the two dimensions. The strain number is placed in the appropriate location for each point on the figure. Data for approximately 5,550 strains belonging to 200 species are shown.

TABLE 3. Variable characteristics of *S. vitulus* strains

Characteristic	No. of strains positive/ no. of strains tested	% of strains positive
Colony diam. of ≤3 mm on P agar	10/11	91
Colony pigment	10/11	91
β-Glucosidase activity[a]	9/11[b]	82
Nitrate reduction	10/11	91
Esculin hydrolysis	7/11 (1/11)[c]	64 (9)[c]
Clumping factor:		
Standard slide test	0/11 (2/11)	0 (18)
Staph Latex kit (Remel)[d]	9/11 (2/11)	82 (18)
Acid produced aerobically from:		
D-Trehalose[b]	0/11 (4/11)	0 (36)
D-Xylose	7/11 (1/11)	64 (9)
D-Cellobiose	2/11 (1/11)	18 (9)
Salicin	1/11 (2/11)	9 (18)
D-Ribose	9/11 (2/11)	82 (18)

[a] Activity determined by the API STAPH-IDENT system.
[b] Most strains that produced a dd 756 pattern were positive for β-glucosidase activity and did not produce acid from mannose or trehalose (the only exception was weak acid production from trehalose by strain DD 6065).
[c] Weak positive reactions were not included as positive reactions. The values in parentheses are the number of strains that exhibited weak positive reactions/ total number of strains tested and the percentage of strains that exhibited a weak positive reaction.
[d] The Staph Latex kit detected both clumping factor and protein A.

Colony growth is reduced or inhibited at 40°C, and none of the strains grows at 45°C. The cells are not motile, and the organisms do not grow anaerobically in thioglycolate semisolid agar. However, they exhibit minimal growth on sheep blood agar and tryptic soy agar plates incubated at 35°C in anaerobic jars. The cells are gram-positive cocci, do not form spores, and occur singly, in pairs, in tetrads, and in clusters.

All strains produce zones of greening on bovine blood agar. All strains are negative for staphylocoagulase, thermonuclease, alkaline phosphatase, pyrrolidonyl arylamidase, ornithine decarboxylase, urease, β-glucuronidase, and β-galactosidase activities, arginine utilization, and acetoin production. All strains are negative for aerobic production of acid from the following substrates: mannose, lactose, galactose, melezitose, xylitol, rhamnose, turanose, arabinose, maltose, *N*-acetylglucosamine, and raffinose. All strains are negative for anaerobic production of acid from glucose and mannitol. All strains are positive for catalase activity, modified oxidase activity, and aerobic production of acid from the following substrates: mannitol, glycerol, sucrose, and fructose. The variable characteristics of *S. vitulus* are shown in Table 3.

The major API profiles are 4200 (six strains) and 4700 (three strains). STAPH Trac profiles exhibit variation with respect to acid production from maltose, raffinose, xylose, and trehalose, nitrate reduction, alkaline phosphatase, and acetoin production, indicating that there is no predominant profile.

***Eco*RI fragments containing rRNA sequences.** The patterns of all *S. vitulus* strains cluster on the basis of similarity and are distinguishable from the patterns of all other bacteria examined, including all previously described *Staphylococcus* species. The species can be recognized by a high level of correlation with the pattern types described in this paper.

TABLE 4. Characteristics that differentiate *S. vitulus* from other novobiocin-resistant, oxidase-positive *Staphylococcus* species[a]

Characteristic	*S. vitulus*	*S. sciuri*	*S. lentus*
Colony size of ≥6 mm on P agar	−	+	−
Anaerobic growth	−	(+)	(±)
Staph Latex kit reaction (Remel)[b]	+	d	d
Hemolysis of bovine blood	±[c]	−	−
Alkaline phosphatase activity	−	+	(±)
β-Glucosidase activity	d	+	+
Esculin hydrolysis	d	+	+
Acid produced aerobically from:			
D-Trehalose	(d)	+	+
D-Mannose	−	(d)	(+)
D-Turanose	−	(±)	(±)
D-Xylose	(d)	(d)	(±)
D-Cellobiose	(d)	+	+
L-Arabinose	−	d	d
Maltose	−	(d)	d
N-acetyl-D-glucosamine	−	d	d
Raffinose	−	−	+

[a] +, 90% or more of the strains are positive; ±, 90% or more of the strains are weakly positive; −, 0 to 10% of the strains are positive; d, 11 to 89% of the strains are positive. Parentheses indicate that a response is delayed.
[b] The Staph Latex kit detected both clumping factor and protein A.
[c] *S. vitulus* produces greening during this test.

Antibiotic susceptibilities. As determined by agar disk diffusion tests, all strains of *S. vitulus* are susceptible to furazolidone, penicillin, erythromycin, and clindamycin. All strains are resistant to bacitracin and novobiocin. Three strains that produce pattern type dd 756 (DD 756[T], DD 4524, and DD 4753) are resistant to tetracycline.

Description of the type strain. The type strain of *S. vitulus* is ATCC 51145 (= DD 756). It has all of the characteristics of the species described above. In addition, it has the properties described below.

Cells are spherical (diameter, 0.9 to 1.1 μm) and occur singly, in pairs, in tetrads, and in clusters.

P agar colonies are raised with ulcerated, irregular centers, irregular edges, and cream pigmentation; the colonies are approximately 2 mm in diameter. Colonies on tryptic soy agar are raised with wide, depressed centers; each colony has a slightly irregular edge, and is pigmented, with a pinkish center blending to a cream edge. These colonies are approximately 10 to 11 mm in diameter. β-Glucosidase is produced. Nitrates are reduced to nitrites. Esculin is not hydrolyzed. The standard slide test is negative for clumping factor. The Staph Latex kit reaction is strongly positive.

Acid is produced aerobically from D-xylose and D-ribose. No acid is produced aerobically from D-cellobiose, salicin, and D-trehalose.

The type strain is resistant to tetracycline.

The guanine-plus-cytosine content of the DNA is 34 mol%.

The type strain produces a type dd 756 pattern when it is studied by using the methods described in this paper.

Distinguishing characteristics. *S. vitulus* can be distinguished from other bacterial species by examining the pattern of restriction fragments containing rRNA sequences that results when the methods described in this paper are used. *S. vitulus* can also be distinguished by its novobiocin resistance, positive oxidase reaction, strong positive Staph Latex kit reaction, absence of alkaline phosphatase activity, and lack of acid production from L-arabinose, maltose, *N*-acetylglucosamine, D-mannose, and raffinose. The major phenotypic features that are useful for distinguishing *S. vitulus* from other novobiocin-resistant, oxidase-positive *Staphylococcus* species are summarized in Table 4.

ACKNOWLEDGMENTS

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Appendix B

Strain Cards Showing Additional Details of Morphological and Physiological Characteristics

Genus: Streptomyces
Species: spp.
Numbers in other collections: DSM 4200

ST 104890

Morphology:

	G	R
ISP 2	good	saffran yellow
	A	SP
	beige red	none
	G	R
ISP 3	good	red orange
	A	SP
	beige red	none
	G	R
ISP 4	good	oxide red
	A	SP
	beige	none
	G	R
ISP 5	good	sand yellow
	A	SP
	none	none
	G	R
ISP 6	good	honey yellow
	A	SP
	none	none
	G	R
ISP 7	good	brown beige
	A	SP
	None	none

Spore chains:
Sporangia: -

Spore surface:
Fragmentation: -

Melanoid pigment: - - - -
NaCl resistance: %
Lysozyme resistance:
pH: Value- Optimum-
Temperature : Value- Optimum- 28 °C
Carbon utilization:

Glu	Ara	Suc	Xyl	Ino	Man	Fru	Rha	Raf	Cel
++	++	+	+	+	+	++	+	+	++

Enzymes:

Gel	Cit	Ure	Arg	Onp	Trp	Lys	Odc	VP	Ind	H2S
+	+	+	+	(+)	-	-	+	-	-	-
2(+)	3-	4+	5-	6+	7+	8+	9+	10+	11+	
12+	13-	14+	15-	16+	17-	18+	19+	20-		

Comments:

Genus: Streptomyces
Species: spp.
Numbers in other collections: DSM 4211

FH 6387

Morphology:

	G	R
ISP 2	good	fawn brown
	A	SP
	beige red	clay brown
	G	R
ISP 3	good	brown beige
	A	SP
	beige red	none
	G	R
ISP 4	good	beige
	A	SP
	grey white	clay brown
	G	R
ISP 5	good	ivory
	A	SP
	none	none
	G	R
ISP 6	good	sand yellow
	A	SP
	none	none
	G	R
ISP 7	good	sand yellow
	A	SP
	none	none

Spore chains:
Sporangia: -

Spore surface:
Fragmentation: -

Melanoid pigment: - - + -
NaCl resistance: %
Lysozyme resistance:
pH: Value- Optimum-
Temperature : Value- Optimum- 28 °C
Carbon utilization:

Glu	Ara	Suc	Xyl	Ino	Man	Fru	Rha	Raf	Cel
+	-	-	-	-	-	-	-	-	+

Enzymes:

Gel	Cit	Ure	Arg	Onp	Trp	Lys	Odc	VP	Ind	H2S
+	+	+	+	-	-	+	(+)	-	-	-
2+	3+	4+	5+	6+	7+	8+	9+	10+	11+	
12+	13-	14-	15-	16+	17-	18+	19+	20-		

Comments:

Genus: Streptomyces
Species: spp.
Numbers in other collections: DSM 4349

FH 6388

Morphology:

	G	R
ISP 2	good	maize yellow
	A	SP
	squirrel grey	none
	G	R
ISP 3	good	sand yellow
	A	SP
	squirrel grey	none
	G	R
ISP 4	good	copper brown
	A	SP
	squirrel grey	none
	G	R
ISP 5	good	sand yellow
	A	SP
	none	none
	G	R
ISP 6	good	sand yellow
	A	SP
	none	beige grey
	G	R
ISP 7	good	sand yellow
	A	SP
	none	none

Spore chains:
Sporangia: -

Spore surface:
Fragmentation: -

Melanoid pigment: - - - -
NaCl resistance: %
Lysozyme resistance:
pH: Value- Optimum-
Temperature : Value- Optimum- 28 °C
Carbon utilization:

Glu	Ara	Suc	Xyl	Ino	Man	Fru	Rha	Raf	Cel
+	+	+	+	+	+	+	+	+	+

Enzymes:

Gel	Cit	Ure	Arg	Onp	Trp	Lys	Odc	VP	Ind	H2S
+	+	+	+		-	-	+	+	-	-
2-	3+	4+	5-	6+	7+	8+	9+	10+	11-	
12+	13(+)	14-	15(+)	16+	17-	18-	19-	20-		

Comments:

Genus: Streptomyces
Species: spp.
Numbers in other collections: DSM 4355

FH 6389

Morphology:

	G	R
ISP 2	good	sand yellow
	A	SP
	gey white	ocher brown
	G	R
ISP 3	good	signal yellow
	A	SP
	grey white	light pink
	G	R
ISP 4	good	nut brown
	A	SP
	grey white	none
	G	R
ISP 5	good	beige
	A	SP
	none	none
	G	R
ISP 6	good	sand yellow
	A	SP
	none	none
	G	R
ISP 7	good	sand yellow
	A	SP
	none	none

Spore chains:
Sporangia: -

Spore surface:
Fragmentation: -

Melanoid pigment: - - - -
NaCl resistance: %
Lysozyme resistance:
pH: Value- Optimum-
Temperature : Value- Optimum- 28 °C
Carbon utilization:

Glu	Ara	Suc	Xyl	Ino	Man	Fru	Rha	Raf	Cel
+	+	+	(+)	+	+	+	+	+	+

Enzymes:

Gel	Cit	Ure	Arg	Onp	Trp	Lys	Odc	VP	Ind	H2S
+	+	+	+	+	-	+	(+)	+	-	-
2+	3(+)	4-	5-	6+	7+	8+	9+	10+	11+	
12+	13+	14+	15-	16+	17+	18+	19+	20-		

Comments:

Genus: Streptomyces
Species: spp.
Numbers in other collections: DSM 13309

ST 101396

Morphology:

	G	R
ISP 2	good	sand yellow
	A	SP
	mouse gey	none
	G	R
ISP 3	good	ivory
	A	SP
	mouse grey	none
	G	R
ISP 4	good	ivory
	A	SP
	mouse grey	none
	G	R
ISP 5	good	light ivory
	A	SP
	none	none
	G	R
ISP 6	good	sand yellow
	A	SP
	none	none
	G	R
ISP 7	good	beige
	A	SP
	none	mahogany brown

Spore chains:
Sporangia: -

Spore surface:
Fragmentation: -

Melanoid pigment: - ++ -
NaCl resistance: %
Lysozyme resistance:
pH: Value- Optimum-
Temperature : Value- Optimum- 28 °C
Carbon utilization:

Glu	Ara	Suc	Xyl	Ino	Man	Fru	Rha	Raf	Cel
+	+	-	-	-	+	+	+	+	+

Enzymes:

Gel	Cit	Ure	Arg	Onp	Trp	Lys	Odc	VP	Ind	H2S
+	+	+	+	+	-	-	+	-	-	-
2+	3+	4+	5-	6+	7+	8+	9+	10+	11+	
12+	13+	14+	15-	16+	17+	18+	19+	20-		

Comments:

Appendix C

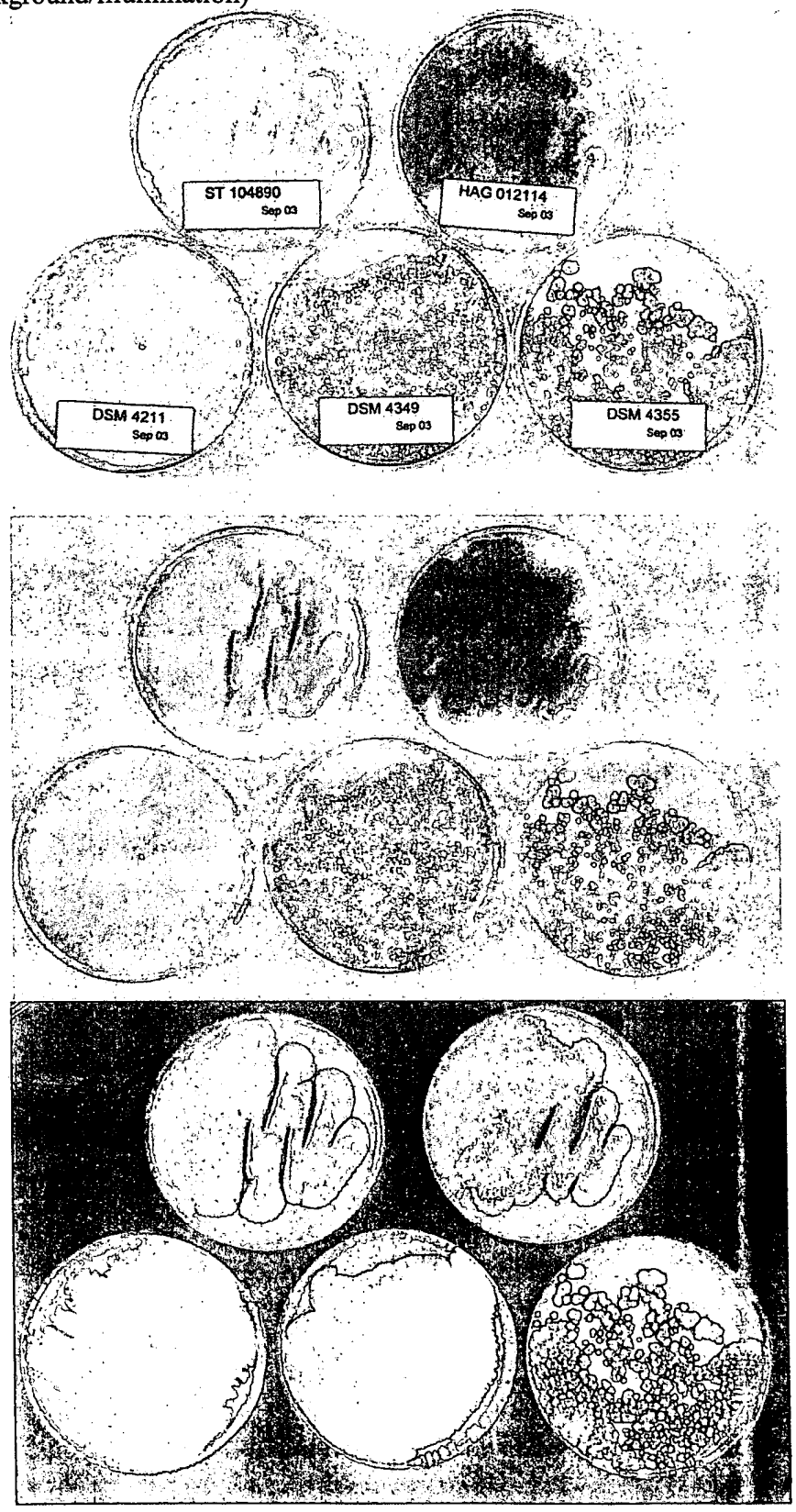
Figures 1 and 2 Showing Colony Color and Growth

Figure 1

Colony color and growth of *Streptomyces spp* DSM 4200 (ST 104890), *Streptomyces spp* DSM 4211, *Streptomyces spp* DSM 4349, *Streptomyces spp.* DSM 4355 and *Streptomyces spp.* DSM 13309 (HAG 012114), grown on ISP 2 for 14 days at 28°C (same experiment, different background/illumination)

Figure 2

Colony color and growth of *Streptomyces spp* DSM 4200 (ST 104890), *Streptomyces spp* DSM 4211, *Streptomyces spp* DSM 4349, *Streptomyces spp.* DSM 4355 and *Streptomyces spp.* DSM 13309 (HAG 012114), grown on ISP 3 for 14 days at 28°C (same experiment, different background/illumination)



Appendix D

Details of the Fatty Acid Analyses

Fatty Acid Analysis:

E039304.57A [2268] st 104890

Volume: DATA File: E039304.57A Seq Counter: 14 ID Number: 2268
Type: Samp Bottle: 12 Method: HMR
Created: 30.09.03 16:15:20
Sample ID: st 104890

Profile:

RT	Response	Ar/Ht	RFact	ECL	Peak Name	Percent	Comment1	Comment2
1,499	1,84E+7	0,018	----	6,944		----	< min rt	
1,523	2,928E+8	0,023	----	6,996	SOLVENT PEAK	----	< min rt	
1,707	4666	0,025	----	7,389		----	< min rt	
1,763	4372	0,020	----	7,508		----	< min rt	
1,840	24916	0,024	----	7,674		----	< min rt	
2,238	1509	0,034	----	8,524		----	< min rt	
2,984	935	0,023	----	10,082		----		
3,650	1973	0,033	----	11,081		----		
6,331	3448	0,034	1,005	13,618	14:0 ISO	8,85	ECL deviates 0,000	Reference 0,008
7,750	3057	0,038	0,984	14,622	15:0 ISO	7,69	ECL deviates 0,001	Reference 0,007
7,884	9240	0,036	0,983	14,712	15:0 ANTEISO	23,20	ECL deviates 0,001	Reference 0,007
8,310	1409	0,038	0,978	14,999	15:0	3,52	ECL deviates -0,001	Reference 0,005
9,051	1976	0,040	0,970	15,458	16:1 ISO H	4,90	ECL deviates -0,003	
9,322	10083	0,040	0,968	15,626	16:0 ISO	24,93	ECL deviates 0,000	Reference 0,005
9,630	3558	0,045	0,965	15,817	16:1 CIS 9	8,77	ECL deviates 0,000	
9,925	5001	0,040	0,962	15,999	16:0	12,30	ECL deviates -0,001	Reference 0,003
11,148	2405	0,042	0,953	16,722	17:0 ANTEISO	5,86	ECL deviates 0,000	Reference 0,003

ECL Deviation: 0,001 Reference ECL Shift: 0,006 Number Reference Peaks: 7
Total Response: 43087 Total Named: 40178
Percent Named: 93,25% Total Amount: 39142

E039304.57A [2288] fh 6387

Volume: DATA File: E039304.57A Seq Counter: 36 ID Number: 2288
Type: Samp Bottle: 32 Method: HMR
Created: 01.10.03 01:13:32
Sample ID: fh 6387

Profile:

RT	Response	Ar/Ht	RFact	ECL	Peak Name	Percent	Comment1	Comment2
1,501	1,727E+7	0,018	----	6,946		----	< min rt	
1,526	2,987E+8	0,024	----	6,999	SOLVENT PEAK	----	< min rt	
1,653	2831	0,021	----	7,271		----	< min rt	
1,703	2991	0,023	----	7,376		----	< min rt	
1,767	4771	0,021	----	7,513		----	< min rt	
1,819	3163	0,021	----	7,623		----	< min rt	
1,845	6602	0,025	----	7,679		----	< min rt	
2,014	5034	0,020	----	8,040		----	< min rt	
2,396	1478	0,024	----	8,852		----	< min rt	
3,658	1278	0,034	----	11,083		----		
5,114	1253	0,031	1,029	12,613	13:0 ISO	0,55	ECL deviates 0,001	Reference 0,004
6,340	20649	0,033	1,003	13,617	14:0 ISO	8,82	ECL deviates -0,001	Reference 0,001
6,839	2260	0,037	0,994	14,000	14:0	0,96	ECL deviates 0,000	Reference 0,002
7,762	16379	0,037	0,982	14,622	15:0 ISO	6,85	ECL deviates 0,001	Reference 0,003
7,895	51000	0,038	0,980	14,712	15:0 ANTEISO	21,28	ECL deviates 0,001	Reference 0,003
8,321	5808	0,038	0,975	15,000	15:0	2,41	ECL deviates 0,000	Reference 0,001
9,061	12440	0,039	0,968	15,457	16:1 ISO H	5,13	ECL deviates -0,004	
9,334	50492	0,039	0,965	15,626	16:0 ISO	20,75	ECL deviates 0,000	Reference 0,001
9,641	13473	0,043	0,962	15,816	16:1 CIS 9	5,52	ECL deviates -0,001	
9,936	20759	0,042	0,960	15,999	16:0	8,48	ECL deviates -0,001	Reference -0,001
10,641	5343	0,044	0,955	16,415	16:0 9? METHYL	2,17	ECL deviates -0,001	
10,827	10539	0,044	0,953	16,525	17:1 ANTEISO C	4,28	ECL deviates 0,000	
11,004	6650	0,042	0,952	16,630	17:0 ISO	2,69	ECL deviates 0,001	Reference 0,000
11,160	16251	0,042	0,951	16,722	17:0 ANTEISO	6,58	ECL deviates 0,000	Reference 0,000
11,279	2460	0,044	0,950	16,792	17:1 CIS 9	0,99	ECL deviates 0,000	
11,632	4510	0,044	0,948	17,001	17:0	1,82	ECL deviates 0,001	Reference 0,000
13,363	1816	0,044	0,937	18,000	18:0	0,72	ECL deviates 0,000	Reference -0,002

ECL Deviation: 0,001 Reference ECL Shift: 0,002 Number Reference Peaks: 12
Total Response: 243361 Total Named: 242083
Percent Named: 99,47% Total Amount: 234897

E039304.57A [2287] fh 6388

Volume: DATA File: E039304.57A Seq Counter: 35 ID Number: 2287
Type: Samp Bottle: 31 Method: HMR
Created: 01.10.03 00:49:04
Sample ID: fh 6388

Profile:

RT	Response	Ar/Ht	RFact	ECL	Peak Name	Percent	Comment1	Comment2
1,503	1,819E+7	0,018	----	6,947		----	< min rt	
1,528	2,643E+8	0,024	----	7,000	SOLVENT PEAK	----	< min rt	
1,842	17593	0,027	----	7,670		----	< min rt	
2,941	2097	0,042	1,131	10,001	10:0	0,22	ECL deviates 0,001	Reference 0,014
3,003	2708	0,034	----	10,096		----		
3,662	2531	0,041	----	11,084		----		
4,112	1402	0,027	1,062	11,608	12:0 ISO	0,14	ECL deviates 0,000	Reference 0,008
4,450	2228	0,029	1,049	11,999	12:0	0,22	ECL deviates -0,001	Reference 0,006
4,556	1214	0,030	1,046	12,096	11:0 ISO 3OH	0,12	ECL deviates 0,006	
5,115	4733	0,031	1,029	12,612	13:0 ISO	0,45	ECL deviates 0,000	Reference 0,005
5,212	2016	0,031	1,027	12,701	13:0 ANTEISO	0,19	ECL deviates 0,000	Reference 0,005
5,535	1610	0,032	1,018	12,998	13:0	0,15	ECL deviates -0,002	Reference 0,003
6,341	68171	0,033	1,003	13,617	14:0 ISO	6,37	ECL deviates -0,001	Reference 0,002
6,839	16574	0,035	0,994	13,999	14:0	1,54	ECL deviates -0,001	Reference 0,002
7,762	103471	0,037	0,982	14,622	15:0 ISO	9,47	ECL deviates 0,001	Reference 0,003
7,897	155958	0,037	0,980	14,713	15:0 ANTEISO	14,25	ECL deviates 0,002	Reference 0,004
8,108	4679	0,039	0,978	14,855	15:1 B	0,43	ECL deviates -0,001	
8,322	32185	0,037	0,975	15,000	15:0	2,93	ECL deviates 0,000	Reference 0,002
9,061	31643	0,041	0,968	15,457	16:1 ISO H	2,85	ECL deviates -0,004	
9,336	176077	0,040	0,965	15,627	16:0 ISO	15,84	ECL deviates 0,001	Reference 0,002
9,643	147745	0,041	0,962	15,817	16:1 CIS 9	13,26	ECL deviates 0,000	
9,940	155546	0,041	0,960	16,000	16:0	13,92	ECL deviates 0,000	Reference 0,001
10,643	47024	0,042	0,955	16,416	16:0 9? METHYL	4,18	ECL deviates 0,000	
10,827	24184	0,043	0,953	16,524	17:1 ANTEISO C	2,15	ECL deviates -0,001	
11,005	21432	0,043	0,952	16,630	17:0 ISO	1,90	ECL deviates 0,001	Reference 0,001
11,160	44257	0,041	0,951	16,721	17:0 ANTEISO	3,92	ECL deviates -0,001	Reference 0,000
11,280	20475	0,043	0,950	16,792	17:1 CIS 9	1,81	ECL deviates 0,000	
11,441	9367	0,051	0,949	16,888	17:0 CYCLO	0,83	ECL deviates 0,000	Reference 0,000
11,633	18117	0,042	0,948	17,001	17:0	1,60	ECL deviates 0,001	Reference 0,001
11,886	1797	0,047	0,946	17,147	16:0 ISO 3OH	0,16	ECL deviates 0,002	
12,440	2481	0,048	0,943	17,466	18:1 ISO H	0,22	ECL deviates 0,006	
12,530	2437	0,050	0,942	17,517	16:0 3OH	0,21	ECL deviates -0,003	
12,963	2144	0,047	0,940	17,767	18:1 CIS 9	0,19	ECL deviates -0,002	
13,057	2485	0,045	0,939	17,821	Sum In Feature 7	0,22	ECL deviates -0,001	18:1 CIS 11/t 9/t 6
13,365	2754	0,048	0,937	17,998	18:0	0,24	ECL deviates -0,002	Reference -0,002
----	2485	---	----	----	Summed Feature 7	0,22	18:1 CIS 11/t 9/t 6	18:1 TRANS 9/t6/c11
----	----	---	----	----		----	18:1 TRANS 6/t9/c11	

ECL Deviation: 0,002
Reference ECL Shift: 0,005
Number Reference Peaks: 18
Total Response: 1111543
Total Named: 1106304
Percent Named: 99,53%
Total Amount: 1072624

E039304.57A [2267] fh 6389

Volume: DATA File: E039304.57A Seq Counter: 13 ID Number: 2267
Type: Samp Bottle: 11 Method: HMR
Created: 30.09.03 15:50:51
Sample ID: fh 6389

Profile: TOTAL RESPONSE LESS THAN 40000,0. CONCENTRATE AND RE-RUN.

RT	Response	Ar/Ht	RFact	ECL	Peak Name	Percent	Comment1	Comment2
1,499	1,791E+7	0,018	----	6,947		----	< min rt	
1,524	2,838E+8	0,024	----	7,001	SOLVENT PEAK	----	< min rt	
1,707	11855	0,025	----	7,391		----	< min rt	
1,796	2391	0,024	----	7,582		----	< min rt	
2,015	3930	0,021	----	8,051		----	< min rt	
6,329	1654	0,035	1,005	13,618	14:0 ISO	7,96	ECL deviates 0,000	Reference 0,006
7,748	2971	0,037	0,984	14,621	15:0 ISO	14,00	ECL deviates 0,000	Reference 0,006
7,882	4467	0,038	0,983	14,711	15:0 ANTEISO	21,02	ECL deviates 0,000	Reference 0,006
9,319	6049	0,041	0,968	15,625	16:0 ISO	28,04	ECL deviates -0,001	Reference 0,003
9,924	4405	0,040	0,962	16,000	16:0	20,30	ECL deviates 0,000	Reference 0,003
11,146	1899	0,043	0,953	16,722	17:0 ANTEISO	8,67	ECL deviates 0,000	Reference 0,002

ECL Deviation: 0,000 Reference ECL Shift: 0,005 Number Reference Peaks: 6
Total Response: 21445 Total Named: 21445
Percent Named: 100,00% Total Amount: 20877
Profile: TOTAL RESPONSE LESS THAN 40000,0. CONCENTRATE AND RE-RUN.

Matches:

Library	Sim Index	Entry Name
HMR1 1.00		(No Match)
HMR2 1.00	0,013	Streptomyces-spp.
	0,012	Actinoplanes-spp.

E039304.57A [2281] hag 012114

Volume: DATA File: E039304.57A Seq Counter: 28 ID Number: 2281
Type: Samp Bottle: 25 Method: HMR
Created: 30.09.03 21:57:50
Sample ID: hag 012114

Profile:

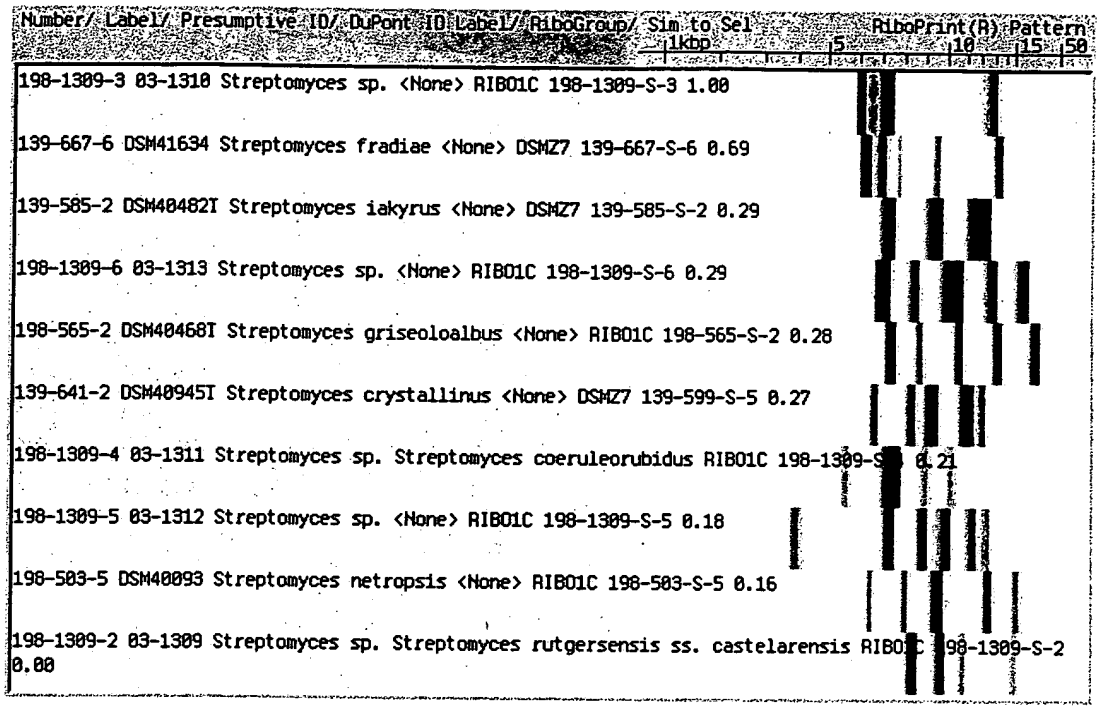
RT	Response	Ar/Ht	RFact	ECL	Peak Name	Percent	Comment1	Comment2
1,501	1,712E+7	0,018	----	6,945		----	< min rt	
1,526	2,929E+8	0,024	----	6,998	SOLVENT PEAK	----	< min rt	
1,653	6142	0,016	----	7,269		----	< min rt	
1,699	2026	0,024	----	7,368		----	< min rt	
1,728	952	0,017	----	7,429		----	< min rt	
1,764	14645	0,019	----	7,507		----	< min rt	
1,817	8614	0,017	----	7,619		----	< min rt	
1,845	12742	0,023	----	7,679		----	< min rt	
2,010	16629	0,019	----	8,032		----	< min rt	
2,140	921	0,013	----	8,309		----	< min rt	
2,392	1149	0,033	----	8,846		----	< min rt	
2,509	1138	0,023	----	9,096		----		
3,036	1791	0,024	----	10,151		----		
3,852	2198	0,040	----	11,307		----		
4,110	1391	0,029	1,061	11,609	12:0 ISO	0,30	ECL deviates 0,001	Reference 0,012
5,113	2676	0,033	1,029	12,612	13:0 ISO	0,56	ECL deviates 0,000	Reference 0,008
5,209	2799	0,034	1,027	12,701	13:0 ANTEISO	0,58	ECL deviates 0,000	Reference 0,008
6,338	43375	0,034	1,004	13,617	14:0 ISO	8,84	ECL deviates -0,001	Reference 0,005
6,453	1439	0,038	1,001	13,705	14:0 ANTEISO	0,29	ECL deviates -0,002	Reference 0,003
6,835	5542	0,035	0,995	13,998	14:0	1,12	ECL deviates -0,002	Reference 0,003
7,759	37082	0,038	0,983	14,622	15:0 ISO	7,41	ECL deviates 0,001	Reference 0,005
7,894	111625	0,038	0,981	14,713	15:0 ANTEISO	22,26	ECL deviates 0,002	Reference 0,006
8,319	7948	0,039	0,976	15,000	15:0	1,58	ECL deviates 0,000	Reference 0,003
9,060	16593	0,041	0,969	15,459	16:1 ISO H	3,27	ECL deviates -0,002	
9,334	117314	0,038	0,967	15,628	16:0 ISO	23,04	ECL deviates 0,002	Reference 0,004
9,640	26209	0,044	0,964	15,817	16:1 CIS 9	5,13	ECL deviates 0,000	
9,838	6369	0,042	0,962	15,940	15:0 ANTEISO 2OH	1,25	ECL deviates 0,004	
9,935	44745	0,039	0,961	16,000	16:0	8,74	ECL deviates 0,000	Reference 0,002
10,641	10344	0,045	0,956	16,416	16:0 9? METHYL	2,01	ECL deviates 0,000	
10,821	13580	0,044	0,955	16,522	17:1 ANTEISO C	2,63	ECL deviates -0,003	
11,000	12019	0,042	0,953	16,628	17:0 ISO	2,33	ECL deviates -0,001	Reference 0,000
11,159	31936	0,042	0,952	16,722	17:0 ANTEISO	6,18	ECL deviates 0,000	Reference 0,001
11,391	3466	0,037	0,951	16,859	16:0 ISO 2OH	0,67	ECL deviates -0,003	
11,439	5166	0,046	0,950	16,887	17:0 CYCLO	1,00	ECL deviates -0,001	Reference 0,000
11,629	3263	0,042	0,949	16,999	17:0	0,63	ECL deviates -0,001	Reference 0,000
13,361	950	0,039	0,939	18,001	18:0	0,18	ECL deviates 0,001	Reference 0,001
18,229	1894	0,041	----	20,832		----	> max rt	

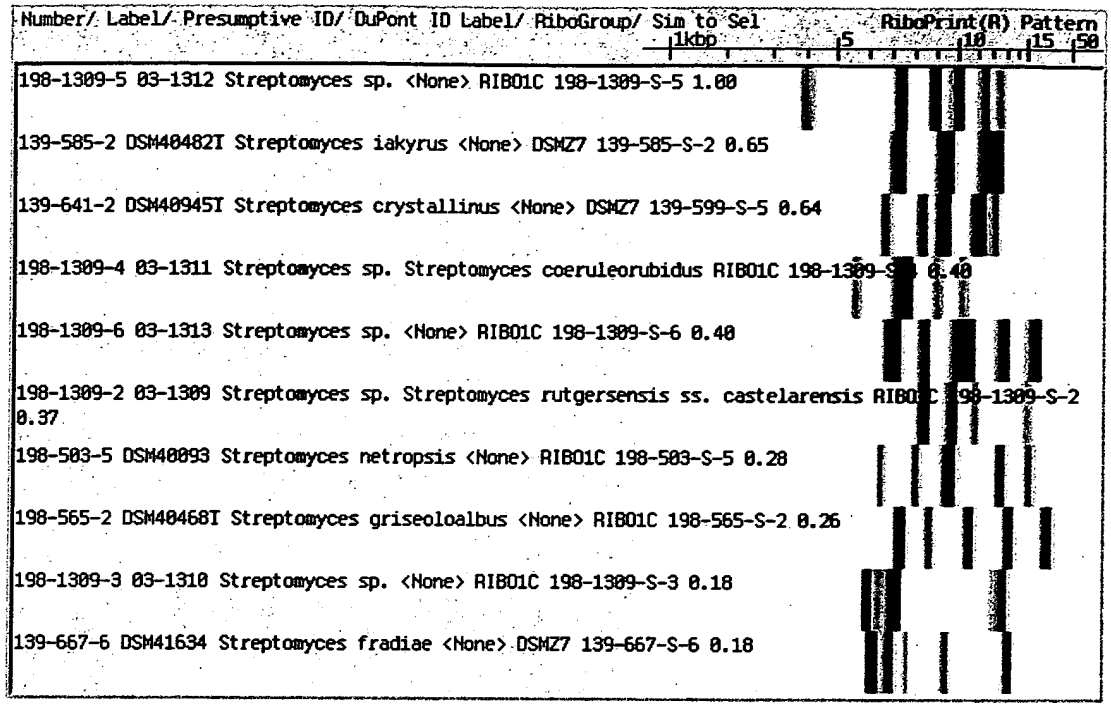
ECL Deviation: 0,002 Reference ECL Shift: 0,005 Number Reference Peaks: 16
Total Response: 510959 Total Named: 505831
Percent Named: 99,00% Total Amount: 492173

= ST 101396

Appendix E

RiboPrints of the Ribotyping Analysis


Number/ Label/ Presumptive ID/ DuPont ID Label/ RiboGroup/ Sim to Sel
RiboPrint(R) Pattern
1kbp
5
10
15
50
198-1309-3 03-1310 Streptomyces sp. <None> RIBO1C 198-1309-S-3 1.00
139-667-6 DSM41634 Streptomyces fradiae <None> DSMZ7 139-667-S-6 0.69
139-585-2 DSM40482T Streptomyces iakyrus <None> DSMZ7 139-585-S-2 0.29
198-1309-6 03-1313 Streptomyces sp. <None> RIBO1C 198-1309-S-6 0.29
198-565-2 DSM40468T Streptomyces griseoloalbus <None> RIBO1C 198-565-S-2 0.28
139-641-2 DSM40945T Streptomyces crystallinus <None> DSMZ7 139-599-S-5 0.27
198-1309-4 03-1311 Streptomyces sp. Streptomyces coeruleorubidus RIBO1C 198-1309-S
0.21
198-1309-5 03-1312 Streptomyces sp. <None> RIBO1C 198-1309-S-5 0.18
198-503-5 DSM40093 Streptomyces netropsis <None> RIBO1C 198-503-S-5 0.16
198-1309-2 03-1309 Streptomyces sp. Streptomyces rutgersensis ss. castelarensis RIBO1C 198-1309-S-2
0.00


Number/ Label/ Presumptive ID/ DuPont ID Label/ RiboGroup/ Sim to Sel
RiboPrint(R) Pattern
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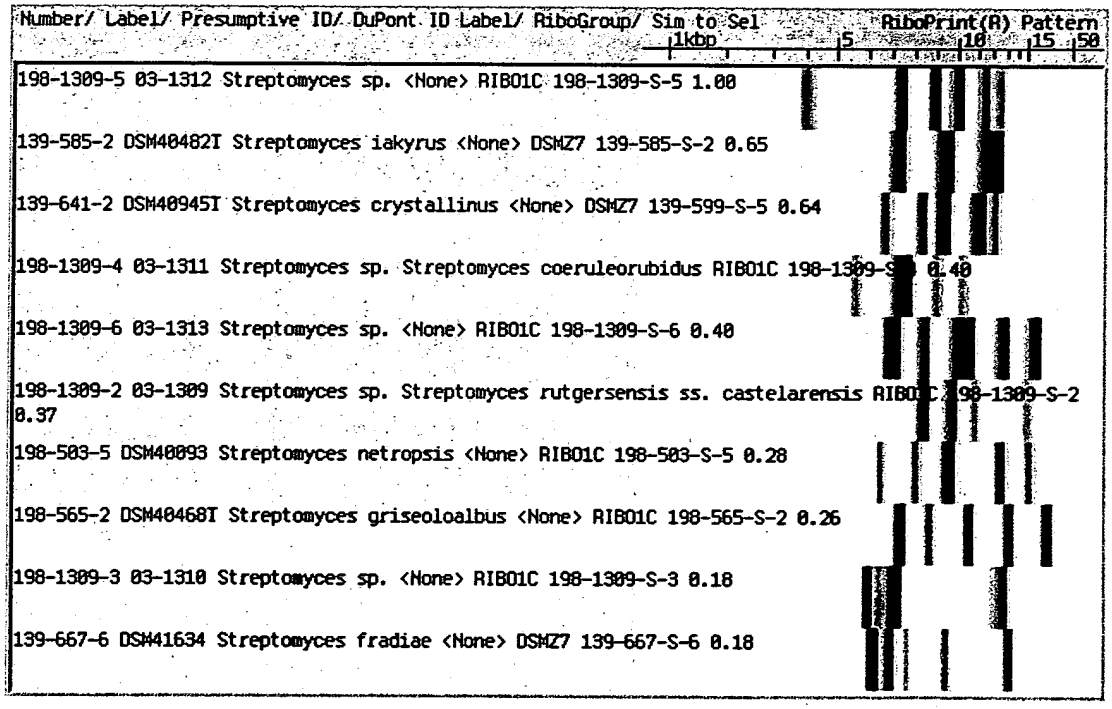
5
10
15
50
198-1309-5 03-1312 Streptomyces sp. <None> RIBO1C 198-1309-S-5 1.00
139-585-2 DSM40482T Streptomyces iakyrus <None> DSMZ7 139-585-S-2 0.65
139-641-2 DSM40945T Streptomyces crystallinus <None> DSMZ7 139-599-S-5 0.64
198-1309-4 03-1311 Streptomyces sp. Streptomyces coeruleorubidus RIBO1C 198-1309-S-4 0.40
198-1309-6 03-1313 Streptomyces sp. <None> RIBO1C 198-1309-S-6 0.40
198-1309-2 03-1309 Streptomyces sp. Streptomyces rutgersensis ss. castelarensis RIBO1C 198-1309-S-2 0.37
198-503-5 DSM40093 Streptomyces netropsis <None> RIBO1C 198-503-S-5 0.28
198-565-2 DSM40468T Streptomyces griseoloalbus <None> RIBO1C 198-565-S-2 0.26
198-1309-3 03-1310 Streptomyces sp. <None> RIBO1C 198-1309-S-3 0.18
139-667-6 DSM41634 Streptomyces fradiae <None> DSMZ7 139-667-S-6 0.18


Number/ Label/ Presumptive ID/ DuPont ID Label/ RiboGroup/ Sim to Sel
RiboPrint(R) Pattern
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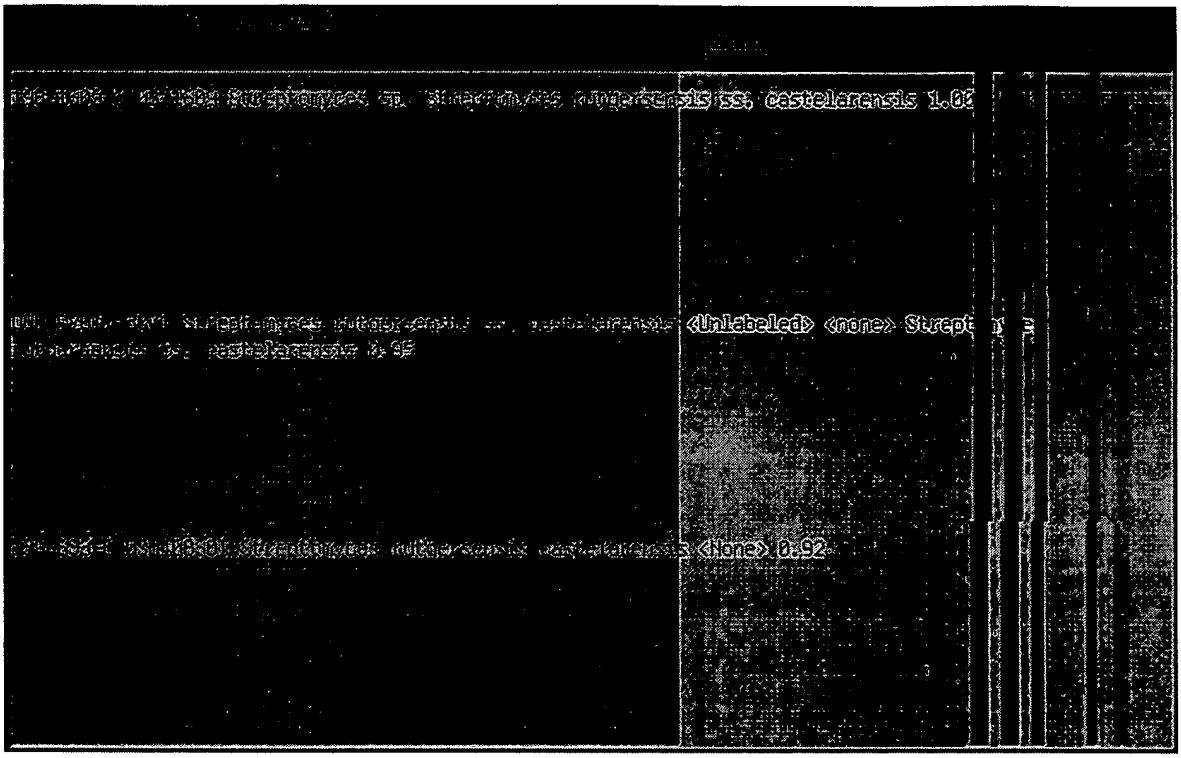
5
10
15
50
198-1309-5 03-1312 Streptomyces sp. <None> RIBO1C 198-1309-S-5 1.00
139-585-2 DSM40482T Streptomyces iakyrus <None> DSMZ7 139-585-S-2 0.65
139-641-2 DSM40945T Streptomyces crystallinus <None> DSMZ7 139-599-S-5 0.64
198-1309-4 03-1311 Streptomyces sp. Streptomyces coeruleorubidus RIBO1C 198-1309-S-4 0.40
198-1309-6 03-1313 Streptomyces sp. <None> RIBO1C 198-1309-S-6 0.40
198-1309-2 03-1309 Streptomyces sp. Streptomyces rutgersensis ss. castelarensis RIBO1C 198-1309-S-2 0.37
198-503-5 DSM40093 Streptomyces netropsis <None> RIBO1C 198-503-S-5 0.28
198-565-2 DSM40468T Streptomyces griseoloalbus <None> RIBO1C 198-565-S-2 0.26
198-1309-3 03-1310 Streptomyces sp. <None> RIBO1C 198-1309-S-3 0.18
139-667-6 DSM41634 Streptomyces fradiae <None> DSMZ7 139-667-S-6 0.18


castelarensis 1.0
<Unlabeled> <none> Strept
<none> 0.92